UNIVERSITY OF ALBERTA

THE EFFECTS OF RE-ORGANIZATION OF CYTOSKELETON AND MATRIX ON GENE EXPRESSION, GROWTH AND APOPTOSIS OF DERMAL FIBROBLASTS

BY

MASOUMEH VAREDI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate studies and Research for acceptance, a thesis entitled **THE EFFECTS OF RE-ORGANIZATION OF CYTOSKELETON AND MATRIX ON GENE EXPRESSION, GROWTH AND APOPTOSIS OF DERMAL FIBROBLASTS** submitted by **MASOUMEH VAREDI** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN EXPERIMENTAL SURGERY**.

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June 23/ 1997

IN THE NAME OF "ALLAH"
THE MOST BENEFICENT & THE MOST MERCIFUL.
PRAISE BE TO "ALLAH", THE SUSTAINER AND THE
CHERISHER OF THE WORLDS.

DEDICATION

To My Family

Fatimeh, Javad, Zahra, Paymun, Paaiam, Hessam, Pooneh and Aava

To My Friends

&

To My Teachers

ABSTRACT

Hypertrophic scars (HSc), which frequently develop as a result of deep dermal injury, are characterized by an extracellular matrix (ECM) of altered composition and organization and activated fibroblasts with altered morphology. I hypothesized that changes in the physicochemical properties of ECM during wound healing and remodeling affect the morphology of the resident fibroblasts and hence their activity.

The morphology of human dermal fibroblasts grown on plastic was altered by detachment with trypsin or EGTA followed by replating, or by treatment of confluent cells with cytochalasin C. These treatments all increased the expression of transforming growth factor-beta1 (TGF-ß1), collagenase and tissue inhibitor of metalloproteinases-I (TIMP-1) but had no effect on the expression of fibronectin or procollagen type I, demonstrating that alterations in morphology of fibroblast selectively modulate the activity of genes involved in structure and metabolism of ECM.

Fibroblasts were cultured in three types of collagen matrix (CM): Anchored (ACM), stress-relaxed (St.rCM) or floating (FCM), environments which more closely mimic the *in vivo* situation than does culture on plastic. The expression of TGF-ß1 mRNA was elevated in all three when compared to culture on plastic but highest in FCM. Collagenase mRNA was elevated to a similar extent in all three types of CM. Fibroblasts continued to grow in

ACM but proliferated more slowly than on plastic. A reduction in cell number was seen in FCM. Apoptotic cells were detected in St.rCM and FCM but not in ACM or cultures on plastic, indicating that the mechanical status of the matrix, or the cells' ability to re-organize it, can regulate growth and survival of the resident fibroblasts.

This work has revealed new insights into cellular mechanisms which could be involved in the pathogenesis of HSc and may have implications for better control of the fibrotic response.

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LIST OF ABBREVIATIONS

Act. D	actinomycin D
ACM	anchored fibroblast populated collagen matrix
cDNA	complementary deoxyribonucleic acid
Chs	cytochalasin
CM	collagen matrix
CsCl	cesium chloride
FCM	floating fibroblast populated collagen matrix
FPCM	fibroblast populated collagen matrix
Cxm	cycloheximide
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGTA	ethylene glycol-bis (ß-aminoethyl)-N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
GITC	guanidinium isothiocyanate
HBSS	Hanks' balanced salt solution
HSc	hypertrophic scar
LAP	latency associated peptide
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Mv1Lu	Mink lung epithelial cell
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
SSC	sodium chloride sodium citrate
TGF-ß	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinases

I

INTRODUCTION

A. OVERVIEW OF WOUND HEALING

Wound healing is (defined as) a series of highly regulated events leading to reconstitution of the integrity of damaged tissue following injury. These events are dependent on each other during the physiologic healing process and involve both cell-cell and cell-matrix/molecular interactions. To orient this overview, the wound healing process is divided into three phases: inflammatory, granulation tissue formation, matrix deposition and remodeling.

Inflammation

Severe injury results in blood vessel disruption and exposure of blood components to the extravascular tissue. The exposure of subendothelial components promotes the adhesion of platelets (to subendothelial components) resulting in aggregation and (triggers) blood coagulation. Immediately after wounding, fibrinogen is converted to fibrin and polymerized fibrin is deposited in the tissue, sealing off the injury and providing an appropriate matrix for migrating cells.

Activated platelets not only trigger blood coagulation, but also release a variety of biologically active compounds from their alpha and dense granules which promote non-specific inflammation. These factors include fibronectin, fibrinogen, thrombospondin, and Von Willebrand factor, which are necessary for platelet aggregation and binding to exposed subendothelial components, platelet derived growth factor (PDGF), transforming growth factor (TGF) -α and -ß which are necessary for recruitment of inflammatory cells and the vasoactive substances such as serotonin, adenosine diphosphate (ADP) and Ca^{++}, which are necessary for vasoconstriction to stop bleeding (Wahl and Wahl, 1992).

Neutrophils are the first defensive cells which arrive in the wound site. They are attracted by a variety of chemotactic factors produced by activated platelets and tissue disruption. Activated neutrophils attack the bacteria which may be present at the wound site by phagocytosis, as well as by secretion of toxic molecules and enzymes. Granular contents of neutrophils released in the extracellular space enhances the degradation of the connective tissue and leads to further tissue damage. The influx of neutrophils seems to be a regulated event in non-specific inflammation since in the absence of bacterial contamination, the influx of neutrophils ceases shortly after the arrival of monocytes.

Having arrived in the inflammatory site, monocytes differentiate into tissue macrophages and increase their phagocytic and pinocytic activities. Moreover, macrophages are an important paracrine source of different growth factors and cytokines including PDGF, tumor necrosis factor (TNF-α), interleukins (ILs), and TGF-ß (Wahl and Wahl, 1992). These factors play important roles in cell migration, cell proliferation, differentiation, angiogenesis and deposition of extracellular matrix (ECM) which are the main events in wound healing. The high level of production of cytokines and growth factors, particularly TGF-ß, at the site of inflammation suggests a significant role for these factors in injured and inflamed tissue (Cromack et al., 1987). In the case of microbial contamination which prolongs the inflammatory phase, the influx and activation of mononuclear leukocytes drive the acute inflammation into the chronic state resulting in further tissue damage and delay in new matrix formation and remodeling.

Granulation Tissue Formation

This phase, also known as the proliferative phase or phase of fibroplasia, is characterized by the migration of fibroblasts and endothelial cells to the wound site and their proliferation. It is now known that cytokines including PDGF, fibroblast growth factor (FGF), TGF-ß, TGF-α, epithelial growth factor (EGF), TNF, and small fragments of matrix components, play important roles in the regulation of this phase of wound healing. Unlike mature scar and normal dermis, granulation tissue is hypercellular and hypervascular and contains high levels of locally-made fibronectin and an elevated level of type III collagen. Fibroblasts deposit a transient loose ECM to fill the wound space. The chemical composition of matrix has a significant effect on the physical properties of tissue which may subsequently modulate morphology, growth and activity of resident cells. Migratory cells use this fibronectin-rich matrix for migration. Migratory endothelial cells divide and differentiate to form the capillary sprouts which finally assemble to form a new vascular network which provides oxygen and nutrients for tissue. Amongst the cytokines, FGF and TGF-α are known to stimulate angiogenesis directly. Although TGF-α mediates all of its biological effects through the EGF receptor, it has been shown that TGF-α is a more potent angiogenic stimulator than EGF *in vivo* (Schreiber et al., 1980). The effect of TGF-ß is paradoxical in angiogenesis. TGF-ß is known as an angiogenic factor *in vivo,* while *in vitro,* it inhibits migration and proliferation of endothelial cells (Orlidge et al., 1989; Tsukada et al., 1995). It seems that, depending on the concentration and the composition and organization of the ECM, TGF-ß modulates the endothelial cell phenotype *in vitro* . Madri et al. (1988) have shown that TGF-ß at a concentration of 0.5 ng/ml or greater induces formation of capillary-like networks of endothelial cells grown in collagen gels but not of endothelial cells grown on plastic.

Wound contraction is another biological event of this phase of wound healing. It is believed that some of the migrating fibroblasts and/or perivascular satellite cells differentiate to myofibroblasts and express filamentous actin in the form of stress-fibers which are arranged parallel to the axis of the cell (Kischer et al., 1982a). The force of contraction has been attributed to these cells (Gabbiani et al., 1972). The role of growth factors such as TGF-ß and PDGF in myofibroblast generation and wound contraction has been studied. Pierce et al. (1989) reported that both TGF-ß and PDGF stimulate granulation tissue formation but not myofibroblast generation in an open wound. Using light and electron microscopic examination of biopsies taken from an open wound model, described by Mustoe et al. (1991), they reported that myofibroblasts were not detected in the growth factor treated wound at 10 days, but 12% of the fibroblasts in the control wound were myofibroblasts at this time. They concluded that acceleration and enhanced granulation tissue formation induced by these growth factors may reduce the need for myofibroblast formation and subsequent wound contraction. Interestingly, they detected collagen fibers in TGF-ß treated wounds but not in PDGF treated and untreated control wound at this time. These findings suggest that TGF-ß may play a significant role in the remodeling and transition of granulation tissue to mature scar. It has been shown that fibroblasts from granulation tissue vary in morphology, cytoskeletal proteins, gene expression, and response to growth factors (Gabbiani et al., 1972; Skalli et al., 1989, Finesmith et al., 1990).

Matrix Deposition and Remodeling

The final phase of wound healing is the remodeling and evolution of granulation tissue. Fibroblasts are believed to be predominantly responsible for this phase. This phase is characterized by changes in the composition and architecture of the ECM and a decrease in the number of cellular elements of granulation tissue such as fibroblasts and endothelial cells. Programmed cell death (apoptosis) has been shown to be involved in the elimination of the expanded fibroblast and endothelial cell populations (Desmouliere and Gabbiani, 1996). As the matrix matures, fibronectin and hyaluronic acid are replaced by fibrous bundles of collagen and proteoglycans. Alterations of the chemical composition of the matrix in turn change the physical characteristic of the matrix and activity of fibroblasts, resulting in the formation of scar tissue. During the remodeling of scar tissue, rearrangement and reorientation of newly synthesized collagen fibers leads to the formation of a strong mature scar in which both organization of fibrous elements and the proportion of matrix components differ from normal dermis. As in the other phases of wound healing, cytokines appear to affect remodeling of granulation tissue and scar formation. The indispensable role of fibrogenic growth factors in the regulation of matrix deposition during wound healing supports the idea that aberration in the expression of these growth factors, and/or abnormal responses to them, play an important role in the outcome of tissue repair process. Amongst them TGF-ß is believed to be prominent.

B. TRANSFORMING GROWTH FACTOR-BETA (TGF-ß)

TGFs-ß are products of a subset of a large gene family that is conserved from *Drosophila* to human (Padgett et al., 1987). Several other structurally related gene products including inhibins and activins, mullerian inhibitory substance, decapentaplegic gene product, product of Vg1 mRNA and several isoforms of bone morphogenic proteins have been identified. Thus far, five isoforms of TGF-ß have been isolated in vertebrates and three isoforms have been identified in mammals. Although TGF-ß was defined initially for its ability to induce a reversible phenotypic transformation of normal rat kidney fibroblast (Roberts et al., 1981), it is now well-documented that TGF-ßs are multifunctional secretory proteins that are involved in the regulation of cell growth and death, differentiation, modulation of immune responses and regulation of matrix formation, events which are important in many physiological processes including wound healing. In this overview the term TGF-ß is used as a generic term where the precise form(s) is (are) not specified.

Chemical Structure and Processing

In 1983 transforming growth factor beta (TGF-ß) was purified from human platelets, human placenta and bovine kidney (Assoian et al., 1983; Frolik et al. 1983). Using human TGF-ß1 with a purity of ≥ 95%, Derynck et al. (1985) showed that non-reduced TGF-ß migrates as a 25 kDa protein in a SDS polyacrylamide gel and reduction with ß-mercaptoethanol shifts the molecular weight to 12.5 kDa. Moreover they confirmed that TGF-ß consists of two identical disulfide-linked monomers. Using direct protein sequencing and cDNA cloning, they established that the C-terminal segment of the 390 amino acid precursor is processed to the 112 amino acid fragment which represents monomeric TGF-ß. TGF-ß is a highly acid resistant polypeptide. Since its full activity is retained in 1 M acetic acid at 95° C, the acid ethanol extraction is an appropriate method to isolate this growth factor from tissues and cell cultures.

It has been shown that the sequences of mature processed TGF-ß1 from human, swine, and bovine sources are identical and differ by only one amino acid residue, the substitution of serine for an alanine at position 75, from those of murine TGF-ß (Derynck et al., 1986). Although TGF-ß2 and TGF-ß3 seem to be less abundant than TGF-ß1, some evidence shows that certain cell types secrete predominantly one of these isoforms (Ikeda et al., 1987). The mature processed forms of TGF-ß2 and TGF-ß3 display a 70% to 80% sequence identity with TGF-ß1 (Derynck et al., 1985; 1987; Marquardt et al., 1987). Very recently, Hinck et al. (1996) have reported the detailed three-dimensional structure of TGF-ß1. In spite of similarity in the backbone of TGF-ß1 and TGF-ß2, several notable differences have been discovered in positional structure and flexibility of the molecules that may be related to differences in receptor binding and biological activities of the isoforms. In contrast to mature forms, the precursor sequences show greater differences, however, particular regions within the precursor are preserved. Interestingly, the arginine-glycine-aspartic acid (RGD) sequence, a general cell recognition signal sequence of the major ECM components, is present in both TGF-ß1 and TGF-ß3 precursors but not in TGF-ß2. Also, three cysteine residues in the proregion of the TGF-ß precursor at position 33, 223, and 225 are highly conserved. Brunner et al. (1989) showed that these cysteine residues are involved in dimerization of the precursor proregion. The role of these cysteine residues in the latency of TGF-ß will be discussed.

Nascent TGF-ß undergoes several post-translational processing events (Figure I-1). The 29 amino-acid hydrophobic signal sequence is cleaved at the Gly-Leu peptide linkage resulting in pro-TGF-ß (Gentry et al., 1988). In addition, oligosaccharide units are added to pro-TGF-ß at three potential glycosylation sites within the amino-terminal portion of the molecule. During transit through the Golgi complex further processing (mannose-6-phosphorylation) produces a phosphorylated glycoprotein containing complex type sialyated N-linked oligosaccharides (Purchio et al, 1988). Since mannose-6-phosphate is a marker that cells use to sort lysosomal proteins intracellularly, the phosphorylated sugars on the TGF-ß precursor may be important for intracellular sorting. However, Dennis and Rifkin (1990) have shown that they may be involved in the cellular activation of the latent form of TGF-ß. Although the biological significance of glycosylation and phosphorylation of pro-TGF-ß needs to be further studied, it has been shown that the carbohydrate moieties within the precursor sequence are essential for the latency of TGF-ß released by cells in culture (Miyazono et al., 1989).

At some stage during synthesis or transit, the TGF-ß monomer is cleaved from the pro-TGF-ß at an Arg-Arg dipeptide (dibasic cleavage site, amino acid residue 287 and 288) resulting in the amino-terminal glycopeptide, which is known as the latency associated peptide (LAP). It has been confirmed that this dibasic cleavage sequence is located within a hydrophilic region. Therefore, it is accessible to a trypsin-like protease (Gentry et al., 1988).

Evidence provided by Miyazono at al. (1988), Wakefield et al. (1988) and Okada et al. (1989) confirmed that the disulfide-linked mature TGF-ß homodimer released by platelets during thrombin-induced degranulation remains non-covalently associated with a disulfide-linked dimer of the LAP to form the " Latent TGF-ß complex". In an elegant study Brunner et al. (1989) showed that site-directed mutagenesis of cysteine residues 223 and 225 in the precursor molecule prevents the dimerization of the precursor proregion resulting in synthesis of a monomeric precursor and biologically active form of TGF-ß by transfected COS cells. Therefore, they concluded that dimerization of the precursor proregion may be necessary for latency of the TGF-ß molecule. In 1987 O'Connor-McCourt and Wakefield reported that the biological latency of TGF-ß in serum is due to the interaction with α–2-macroglobulin, a serum protease inhibitor. One year later this group (Wakefield et al., 1988) showed that the latent form of platelet TGF-ß cannot bind to α–2-macroglobulin, but that the activated form of TGF-ß does bind. Based on these observations they suggested that α–2-macroglobulin scavenges excess active TGF-ß from the extracellular fluid and thereby limits the systemic effects of this growth factor and confines its action to those target tissues capable of activating it.

Gene, Promoter and Regulatory Elements

The genes and cDNAs for mammalian TGF-ß1, TGF-ß2, and TGF-ß3 have been cloned and sequenced (Derynck et al., 1985, 1986; Marquardt et al., 1987; De Martin et al., 1987). The loci of these genes have been identified in the human on chromosomes 19, 1, and 14 respectively (Fujii et al., 1986; Barton et al., 1988). According to Derynck et al. (1987), the human TGF-ß1 gene has been estimated to span more than 100 Kb and is divided into 7 exons and 6 introns (Figure I-2). The coding sequence corresponding to the 390 amino acid human TGF-ß1 (hTGF-ß) precursor, pre-pro TGF-ß, has been also deduced.

Kim et al. (1989a) have sequenced and characterized the 5′ flanking region of the TGF-ß1 gene. They have identified several regulatory regions including two different promoter regions, one region with enhancer-like activity and two negative regulatory regions (Figure I-2, A). Apparently, the negative regulatory regions correspond to fat-specific element 2 (FSE2) and interferon responsive element (IRE) and the positive regulatory regions possess several binding sites for putative transcription factors such as nuclear factor 1 (NF-1) SP1 and activator protein-1 (AP-1) complexes. In contrast to the coding region, the promoter region of the mammalian TGF-ß genes exhibit minor similarity in sequence indicating differential regulation of TGF-ß isoforms. Using an SP1 expression plasmid in a cell background devoid of any SP1 homology (*Drosophila melanogaster* cell culture system), Geiser et al. (1993) have shown that TGF-ß1 and TGF-ß3 promoters were responsive to the SP1 transcription factor while the TGF-ß2 promoter was completely unresponsive.

In spite of intensive investigation in defining the biological activities of TGF-ß, the mechanism(s) by which expression of this multifunctional growth factor is regulated is not yet fully understood. Apparently, expression of TGF-ß1 is largely governed by the AP-1 complex, a heterodimer of *c-fos* and *c-jun* or a homodimer of *c-jun* protooncoproteins. In 1988, Obberghen-Schilling et al. reported that TGF-ß1 increases its own expression in both normal and transformed cells. Later, Kim et al. (1989b) showed that the autoinduction of TGF-ß1 is mediated through the AP-1 binding elements. Moreover, the repression of 12-o-tetradecanoylphorbol-13- acetate (TPA)-mediated TGF-ß1 induction by the members of the nuclear receptor family such as retinoic acid receptor -α, -ß and retinoid X receptor-α has been reported (Salbert et al., 1993).

Activation

Transforming growth factor beta is secreted by many transformed and non-transformed cells in a latent form. The latent form of TGF-ß is not able to bind to its cellular receptor and cannot initiate signal transduction. Therefore, it is biologically inert. Apparently the receptor binding site and some other TGF-ß epitopes are masked by its LAP (Derynck et al., 1985; Lawrence et al., 1985; Lyons et al., 1988; Wakefield et al., 1988).

TGF-ß is a widely distributed protein in animal tissue with diverse biological activities. Moreover, most cells express the receptor for TGF-ß. Since many cells that synthesize TGF-ß are able to respond to it, it is conceivable that TGF-ß can act as an autocrine and/or paracrine factor in the regulation of cell function. Therefore, the local activation of this growth factor in the vicinity of special target cells probably plays a central role in the regulation step of the biological effects of TGF-ß.

In 1985 Lawrence and co-workers found that four different treatments, acidification (pH 6-2.5), alkalinization (pH 8.8-11.9), exposure to urea (8 M), and heating at 100° C for 3 minutes, significantly increased the activity (stimulation of anchorage-independent growth) of latent TGF-ß released by chicken embryo fibroblast on normal rat kidney fibroblast (NRK-49F) cells. Lyons et al. (1988) also reported the effect of acidification on the activation of latent TGF-ß released by NRK-49F. Also, Pircher and colleagues (1986) confirmed that the acid-ethanol extract of TGF-ß from human blood platelets is about 300 fold more active in inducing the anchorage-independent growth of NRK-49F cells in the presence of epidermal growth factor (EGF) than the neutral extract prepared by sonication of platelets in PBS at neutral pH.

Although Lawrence's, Lyons's and Pircher's work have provided valuable information regarding the activation of TGF-ß in *in vitro,* these conditions are unlikely to be mechanisms involved in the activation of this growth factor *in vivo*. The exact pathophysiological significance of these observations remains to be determined. However, it could be speculated that within a particular condition (wounded or remodeling tissue) the latent TGF-ß may become activated by some cell products (enzymes) and/or an adequately acidic microenvironment.

Cell-associated proteases have been considered likely candidates in the activation of latent TGF-ß. Activation of the TGF-ß complex in the presence of some proteases has been studied. Lyons et al. (1988) demonstrated that plasmin, a broad spectrum protease sometimes associated with the cell membrane, can activate the latent TGF-ß released by NRK-49F cells and AKR-MCA mouse fibroblasts in culture. Using a radioreceptor and soft agar assay, they found that plasmin-treated conditioned medium was quantitatively similar in activity to that of mild acid-treated conditioned medium (pH 4.4-5.5). Since extremes of pH generated TGF-ß activity that was significantly greater than the mild acid-generated and/or plasmin-generated activity, they concluded that conditioned medium may contain at least two different pools of latent TGF-ß. One pool is mild acid-resistant and needs harsher acidic or basic pH and cannot be activated by plasmin, while the second pool can be activated by mild acidic or basic pH and/or plasmin.

A series of sophisticated studies by Orlidge et al. (1987, 1989) and Sato and Rifkin (1989) further support the activation of TGF-ß by plasmin in the tissue. Based on the evidence which shows a central role for pericytes in the regulation of endothelial cell growth, they showed that direct coculture of pericytes with capillary endothelial cells or smooth muscle cells with endothelial cells completely inhibited endothelial cell proliferation. They also showed that serum-free conditioned medium from each cell line alone had no effect on endothelial cell growth and contained latent TGF-ß. These observations suggest that pericyte-endothelial cell interaction is essential in the activation of TGF-ß in this particular coculture system. The role of plasmin in this coculture system was also studied. Sato and Rifkin measured the activity of TGF-ß by its inhibitory effect on endothelial cell migration. They showed that bovine aortic endothelial (BAE) cell migration was inhibited when cocultured with pericytes but not when each of the cell types were cultured separately and that the addition of anti-TGF-ß totally neutralized the inhibition mediated by the pericytes in the cocultures. They examined whether plasmin mediated the conversion of the latent TGF-ß to an active form in this coculture system. They showed that treatment of cocultures with serine protease inhibitors such as aprotinin, which is a broad spectrum serine protease inhibitor and $\alpha 2$- plasmin inhibitor, which is a rather specific plasmin inhibitor, eliminated the effect of pericytes on BAE cell migration and bovine anti-urokinase plasminogen activator (PA) antibody also blocked the effect. In addition to cell membrane associated plasmin, endothelial cells are able to produce metalloproteinases. Unfortunately, little if any information about the role of tissue specific metalloproteinases in the activation of TGF-ß is available. However, it is possible that tissue specific urokinases and metalloproteinases are involved in the activation of TGF-ß *in vivo*. Lyons et al. (1988) have suggested a negative feedback of plasmin-mediated activation of TGF-ß. The sequence of events that regulate TGF-ß activation by plasmin is postulated as follows: Plasminogen is converted to plasmin by tissue plasminogen activator (urokinase) action. Subsequently, the inactive form of TGF-ß is proteolytically activated and in turn can bind to specific cell membrane receptors and increase the plasminogen activator inhibitor (PAI) and decreases the urokinase level resulting in a negative feedback on TGF-ß activation and consequently TGF-ß action.

Endoglycosidases are another candidate for activation of TGF-ß. Miyazono et al. (1989) reported that the removal of N-linked carbohydrate complex of latent TGF-ß from human platelets by endoglycosidase F (Endo F), led to the release of the active form of TGF-ß. Furthermore, they showed that sialic acid residues in the precursor are involved in the latency of TGF-ß, since sialidase also activated the latent form of this growth factor.

The possibility that ECM components regulate TGF-ß activity is intriguing. Proteoglycans which are extracellular components of various connective tissues, have been considered as modulators of many growth factors (Ruoslahti et al., 1991). It has been suggested that the TGF-ß binding proteoglycan, betaglycan, one of the three receptors for TGF-ß, is a non signaling binding protein which may act as a "reservoir" of TGF-ß near the cell surface. Yamaguchi et al. (1990) reported that TGF-ß binds to a small ECM proteoglycan, decorin, through the core protein which is leucine-rich and is associated with type I collagen fibrils in tissues. Decorin not only binds TGF-ß but also blocks its activity. Interestingly, Hausser et al. (1994) reported that the formation of TGF-ß-decorin complex only neutralized distinct activities of TGF-ß. They showed that addition of decorin to osteosarcoma cell cultures inhibited the TGF-ß1-mediated up-regulation of biglycan production but the down-regulation of proteoglycan-100 was unaffected. They have also shown that TGF-ß-mediated stimulation of retraction of fibroblast-populated collagen matrix was not affected by the addition of decorin. It has been reported that thrombospondin, a multidomain glycoprotein, of connective tissue, α–granules of platelets, wound fluid and embryonic tissue, binds and activates recombinant and endothelial cell-derived latent TGF-ß (Schultz-Cherry et al., 1994). Modulation of TGF-ß activity by other circulating molecules such as α–2 macroglobulin and immunoglobulin IgG has been reported (Caver et al., 1996). Although little is known about the significance of these interactions, they may provide the proper three-dimensional structure for the TGF-ß complex resulting in retention or perturbation of latency. Moreover, these interactions may participate in important functions such as pericellular retention, delivery, receptor presentation, and scavenging of the active form of TGF-ß. Very recently, Bottinger et al. (1996) have shown that recombinant LAP is a potent inhibitor of bioactive mammalian TGF-ßs both *in vivo* and *in vitro*. Clearly, finding the mechanism(s) of cellular activation of latent TGF-ß is a crucial step towards understanding the endocrine, paracrine and autocrine action of this multifunctional growth factor.

Receptors and Receptor-Mediated Signaling

With the notable exceptions of human retinoblastoma cells, rat phaeochromocytoma and undifferentiated embryonal carcinoma cells, all cell types display one or more specific high-affinity cell surface receptors for TGF-ß (Massague et al., 1985). Dissociation equilibrium constant ranges from 6×10^{-11} to 10^{-12} M and the number of TGF-ß receptors have been reported to be as low as 200 to as high as 10^5 per cell. Based on the molecular weight of the cross-linked complex, three distinct classes of cell surface TGF-ß binding proteins have been characterized. These include type I or low molecular weight components (50-80 kDa), type II or intermediate components (85-140 kDa), and type III or high molecular weight components (280-310 kDa).

Type I and II receptors are glycoproteins (Figure I-3) containing complex N-linked carbohydrates, which are not essential for cell surface expression or for ligand binding but may affect receptor stability or affinity for TGF-ß. Using radio-iodinated TGF-ß, Frolik et al. (1984) characterized saturable receptors for TGF-ß on NRK fibroblasts. Since other growth factors such as EGF, insulin, insulin like growth factor I and II (IGF I, IGF II), PDGF and TGF-α were not able to compete for the binding of ^{125} I-TGF-ß to the receptors, they confirmed the specificity of the receptors. Wakefield et al. (1987) examined the distribution and modulation of the TGF-ß receptors on a wide variety of cell types. They found that all cell types including normal and neoplastic cells exhibit specific high-affinity receptors for TGF-ß and demonstrated a strong inverse relationship between receptor number and binding affinity. Evidence provided by Laiho et al. (1990a) and Boyd et al. (1989) confirmed the involvement of type I and II receptors in signal transduction for some specific TGF-ß-induced activities. Using different classes of TGF-ß-resistant cell mutants derived from mink lung epithelial cells (Mv1Lu) following chemical mutagenesis, they characterized the TGF-ß receptors in these mutants and showed that in most cases, responses (growth inhibition, morphological changes and up-regulation of PAI-1 expression) of Mv1Lu cells to TGF-ß were completely lost, regardless of whether the cell mutants lacked only the type I receptor, or had low type I receptor levels with a structurally altered type II receptor, or lacked both type I and II receptors. Since in many mutants type I and II receptor were simultaneously lost or altered, they concluded that the type I and II receptors were the active mediators of the TGF-ß biological activities and both receptor components may interact as a part of a common receptor complex. Of significance, in an intensive screening, Massague et al. (1990) reported that among more than 100 cell lines surveyed in their laboratory they have not found any cell line that responds to TGF-ß and lacks the type I receptor.

Type III TGF-ß binding protein is an integral membrane proteoglycan. TGF-ß binds to a site located in the core protein of the type III receptor. The glycosaminoglycan (GAG) chains, consisting of chondroitin sulfate and/or heparan sulfate, are linked to another domain of the core protein distinct from the TGF-ß binding site. Mutants defective in GAG synthesis still express the TGF-ß binding site in the core protein. Also, the growth inhibitory response to TGF-ß is similar in parental cells and in mutants that lack GAG synthesis (Cheifetz & Massague, 1989). Thus, these chains, apparently, are not essential for the binding of TGF-ß to the core protein and biological action of TGF-ß. Moreover, enzymatic removal of GAG chains by chondroitinase and/or heparitinase has no effect on the TGF-ß binding activity (Segarini and Seyedin, 1988). Andres et al. (1989) reported the existence of soluble forms of TGF-ß binding proteoglycans that lack the membrane anchor and showed that these could be extracted from the extracellular matrices and culture media of several cell types and serum. They proposed the name "betaglycan" for this group of TGF-ß binding proteoglycans. As judged by SDS-PAGE, betaglycans are membrane-bound or soluble proteoglycans with a heterogeneous core protein of 100-200 kDa that contain a high affinity binding site for TGF-ß, and carry ~10 kDa of N-linked glycan chains and between 100-300 kDa of heparan sulfate and/or chondroitin sulfate glycosaminoglycans chains. Although the origin of soluble betaglycan and the regulation of its expression is not clear, it could be postulated that it is a product of posttranslational modification, alternative mRNA splicing, or a separate gene. However, Casillas et al. (1991) reported that the extracellular domain of betaglycan has a susceptible cleavage site adjacent to the transmembrane region and can be released as a soluble proteoglycan (Figure I-3).

Although in many cell lines the most abundant receptor for TGF-ß is type III, some evidence exists to suggest that the type III TGF-ß receptor is not necessary for the mediation of TGF-ß action. For example, myoblasts and hematopoietic progenitor cells which are highly responsive to TGF-ß, do not express the type III receptor. In addition, chemically mutagenized TGF-ß-resistant cells that lack the type I receptor still express normal levels of type III receptor with ligand-binding properties similar to the receptor in wild-type cell (Massague, 1990). Although the exact biological role of betaglycan in mediating TGF-ß action is not yet clear, it has been suggested to be involved in the presentation of TGF-ß to the type I and/or type II signaling receptors and may act as a reservoir or clearance system for the active form of TGF-ß.

Many cell types express a 180-kDa homodimeric transmembrane glycoprotein which is known as endoglin (Figure I-3). Endoglin has been shown to be primarily expressed in endothelial cells and to bind TGF-ß1 and ß3 isoforms with high affinity (Lastres et al., 1994). It is believed that endoglin like betaglycan presents the ligands to the type I and/or type II signaling receptors. However, very recently, Lastres et al., (1996) have reported that several cellular responses to TGF-ß1 were totally abrogated in stable transfectants of monocytes overexpressing endoglins with a mutated cytoplasmic domain. This suggests that endoglin may play a more direct role in TGF-ß signaling pathway(s).

Cheifetz and colleagues (1988a) have reported a fourth TGF-ß receptor on the surface of rat pituitary tumor cells (GH3 cells). They found that the type IV receptor also binds inhibin and activin, members of the TGF-ß family that regulate the release of follicle-stimulating hormone from the pituitary gland. Affinity labeling of this TGF-ß binding protein by cross-linking to ^{125}I TGF-ß yielded a 70-74 kDa labeled complex with an average of 2700 TGF-ß binding sites per cell. In contrast to the type IV receptor, they showed that activin and inhibin were not able to compete for binding to either type I, type II, or type III TGF-ß receptors. Whether the type IV receptor can act as a signaling receptor is not yet clear.

A 400 kDa TGF-ß binding protein has been detected in plasma membranes of bovine liver by cross-linking and SDS-PAGE assays. Grady et al. (1991) demonstrated that this high molecular weight TGF-ß receptor is a novel nonproteoglycan and is not related to type I, II, III, or IV TGF-ß receptors. This receptor is designated as a type V receptor of TGF-ß. A variety of cultured cells, including cells reportedly lacking the other types of TGF-ß receptors have been examined. They showed that the type V receptor was expressed in mink lung epithelial and NIH 3T3 cells (which express type I, II and III receptors), basal cell carcinoma 1 cells (which lack the type I, II and III receptors) and baby hamster kidney cells (which lack type II, and III receptors). The specificity of type V TGF-ß receptor for both TGF-ß1 and TGF-ß2 and the contribution in mediating ^{125}I-TGF-ß binding have been investigated. Since this receptor undergoes ligand-induced internalization, one of the properties common to many cell surface receptors, it seems that the type V receptor is a functional receptor.

Most cell lines display three major TGF-ß receptors. However, the relative proportions of these receptors vary in different cell lines. Apparently, there is no correlation with whether the cell lines are derived from normal or tumor tissue or are transformed *in vitro* (Massague et al., 1990). The interaction of TGF-ß receptors with various TGF-ß isoforms has been studied. Type III receptor binds both TGF-ß1 and TGF-ß2 with similar affinities, whereas type I and II receptors have ten-fold higher affinity for TGF-ß1 than for TGF-ß2 (Segarini et al., 1987). However, Cheifetz et al. (1990) detected a subset of type I and II receptors in Mv1Lu and BRL-3A (newborn rat liver) cells that have a higher affinity for TGF-ß2. Taking advantage of the TGF-ß1.2 heterodimer from porcine platelets, Cheifetz et al. (1988b) showed that the presence of one ß1 chain in TGF-ß1.2 improves its affinity for type I and II receptors and makes it a more potent agonist than TGF-ß2 homodimers. Based on this observation they concluded that individual TGF-ß chains contribute to the receptor binding of TGF-ß.

Several factors have been shown to regulate expression of TGF-ß receptors. Differentiation, increased cell density and exposure to physiological or saturating concentrations of TGF-ß, have been shown to induce a down-regulation of receptor number (Ewton et al., 1988; Rizzino et al., 1988; Frolik et al., 1984). The effect of retinoic acid is dependent on the cell system. Retinoic acid has very little effect on receptor binding in NRK cells, while in embryonal carcinoma cells, retinoic acid up-regulates TGF-ß binding (Wakefield et al., 1987; Rizzino, 1987). Growth factors and TPA have been shown not to have significant effects on TGF-ß binding.

Little is known about the intracellular pathway by which the TGF-ß signal is transduced. Several second messenger system have been reported for TGF-ß-mediated biological activities. Murthy et al. (1988) and Howe et al. (1990) demonstrated that negative and positive growth activities of TGF-ß are mediated through the G protein-linked signaling pathways. In 1992, Lin et al. reported that the cytoplasmic domain of the type II TGF-ß receptor is a Ser/Thr kinase domain and acts as an important mediator of TGF-ß signal transduction. It is now accepted that type I and type II receptors are both transmembrane Ser/Thr kinases with a small ecto-domain and a relatively big endo-domain. However, the autophosphorylation of the type II receptor on tyrosine, suggests a dual specificity of the kinase activity for this receptor (Lin et al., 1992; Derynck, 1994). It is believed that co-expression of the type I and type II receptors is required for TGF-ß responsiveness. The co-immunoprecipitation of both receptor types following cross-linking of iodinated-TGF-ß suggests that the functional TGF-ß receptor is a heteromeric complex (Figure I-3) of type I and type II receptors (Ebner et al., 1993). It has been suggested that the type II and type I receptors transphosphorylate each other's cytoplasmic domains, which leads to receptor activation and interaction with signaling molecules. Moreover, it has been shown that type I receptor can be phosphorylated by type II receptor (Derynck, 1994). Although there is evidence to show that mothers against decapentaplegic (MAD) proteins are involved in TGF-ß signaling (Shibuya et al., 1996), the pathway(s) that convey the cell surface signal to the nucleus in mammalian cells is (are) not yet characterized.

Biological Activities

Cell Growth Depending on the cell type and the condition and the presence of other growth factors, TGF-ß may have stimulatory, inhibitory, a biphasic effect or no effect on cell proliferation. It is now well-documented that TGF-ß inhibits the growth of almost all non-neoplastic and neoplastic epithelial cells in culture, including rapidly growing skin keratinocytes or quiescent keratinocytes stimulated by addition of EGF (Coffey et al., 1988). Silberstein and Daniel (1987) demonstrated that implantation of a TGF-ß slow-release pellet adjacent to mouse mammary epithelial end buds significantly inhibited ductal growth. The mechanism of growth inhibition of epithelial cells by TGF-ß has been studied. Coffey et al. (1988) indicated that the effects of TGF-ß on the expression of c-myc mRNA may be a key point in the growth-inhibitory response to TGF-ß since TGF-ß rapidly reduces *c-myc* mRNA and protein. Moreover, it has been shown that the protein product of the retinoblastoma gene (pRB) is involved in the TGF-ß pathway for suppression of *c-myc* transcription (Laiho et al., 1990b). TGF-ß is also a potent inhibitor of the growth of endothelial, lymphoid and myeloid cells. Furthermore, Russell et al. (1988) showed that intravenous injection of TGF-ß1 and TGF-ß2 inhibited the early phase of hepatocyte proliferation following partial hepatectomy in rat. In addition, Braun et al. (1988) showed that the level of TGF-ß mRNA increases during liver regeneration and reaches a peak after the initial wave of hepatocyte mitosis. They proposed that the induction of TGF-ß might be a negative feedback regulation to prevent further liver growth.

Evidence such as localization of TGF-ß during embryogenesis to regions of mesenchymal re-organization (Heine et al., 1990), the enhancing effects of TGF-ß in wound healing (Quaglino et al., 1990) and bone formation (Rosen et al., 1988), and stimulation of DNA synthesis in early passage human fetal fibroblasts (immature fibroblasts; Hill et al., 1986), suggest that TGF-ß stimulates the growth of mesenchymal cells. The mechanism of this effect is not yet clear. However, the cell-cycle kinetics of TGF-ß stimulation of fibroblastic cell proliferation are unique in that G1 is prolonged by about 12 h when compared to G1 in other growth factor-stimulated cell types. Leof and co-workers (1986) demonstrated that TGF-ß initially induced expression of the PDGF B chain mRNA, the gene product of the proto-oncogene *c-sis*, in AKR-2B fibroblasts and increased PDGF expression. PDGF is a mitogen for these cells. It has been reported that TGF-ß has no significant effect on human dermal fibroblasts in confluent cultures (Varga et al., 1987). However, TGF-ß activates monocytes to secrete factors mitogenic for fibroblasts. On the basis of these results, TGF-ß may be considered an indirect mitogen for fibroblastic cells.

Extracellular Matrix Formation TGF-ß stimulates the synthesis of major extracellular matrix (ECM) proteins, such as fibronectin, collagen, some of the proteoglycans, glycosaminoglycans and elastin *in vitro* as well as *in vivo*. In 1987 Ignotz et al. showed that TGF-ß strongly stimulates the formation of type I procollagen and fibronectin by increasing the transcription rate and stabilization of transcripts in cultured rat fibroblasts and myoblasts. Varga et al. (1987) reported that TGF-ß stimulates type I and III collagen and fibronectin synthesis at the level of mRNA in normal human dermal fibroblasts. They showed that the effects of TGF-ß are accompanied by an elevated steady-state level of the transcripts which persists for at least 72 hr after removal of TGF-ß from the culture media. It seems, therefore, that TGF-ß induces a long-lasting stimulation of the biosynthesis of ECM component in fibroblasts. The stimulatory effect of TGF-ß on type I, II, and V collagen mRNA in cultured normal and fibrotic adult human lung fibroblasts has also been reported (Raghu et al., 1989). Bassols and Massague (1988) demonstrated that TGF-ß controls the level of expression and molecular structure of dermatan sulfate proteoglycan, the major type of sulfated proteoglycan present in the extracellular matrix and culture medium of various human and other mammalian cell types. Since TGF-ß1 and TGF-ß2 showed similar potency on proteoglycan synthesis, they suggested that these cellular responses may be induced through type III receptors. It has been shown that TGF-ß increases the molecular mass of the glycosaminoglycan chains in various mesenchymal and epithelial cell lines (Bassols and Massague, 1988). Falanga et al. (1987) demonstrated that TGF-ß preferentially stimulates glycosaminoglycan synthesis in scleroderma fibroblasts, a disease of unknown cause that is associated with a progressive fibrosis of connective tissue in the skin and other organs, as compared to normal fibroblasts.

An expanding body of data supports the prominent effects of TGF-ß on extracellular matrix *in vivo*. Subcutaneous injection of TGF-ß in normal mice causes the formation of collagen-containing granulation tissue at the site of injection within 48 h (Roberts et al., 1986). The histologic examination of TGF-ß treated wounds shows an increase in synthesis and deposition of collagen. These wounds have been characterized by an increased number of fibroblasts synthesizing procollagen type I (Pierce et al., 1989). Using an excisional wound model in pig, Quaglino et al. (1990) showed the localized effect of exogenous TGF-ß on extracellular matrix gene expression through the accumulation of mRNA for collagen type I and III, fibronectin and elastin.

Since the extracellular matrix components influence expression of the individual cell phenotype, TGF-ß may also control expression of specific phenotypes by regulating the composition of ECM.

Extracellular Matrix Degradation TGF-ß not only stimulates the expression of ECM components but may also inhibit the degradation of matrix proteins by the regulation of proteinases and proteinase inhibitors that affect the turnover of the ECM proteins. Edwards et al. (1987) reported a significant decrease in collagenase and stromelysin and an increase in tissue inhibitor of metalloproteinases (TIMP) when fibroblasts were treated with TGF-ß. Using the nuclear run-on assay, they showed that these effects were at the level of transcription. Plasmin is another target proteinase for TGF-ß. Plasmin has the ability to degrade a variety of matrix proteins and is known to activate latent collagenase. TGF-ß decreases the synthesis of tissue plasminogen activators while stimulating the synthesis of plasminogen-activator inhibitor (Laiho et al., 1986). Effects of TGF-ß on the regulation of the proteinases *in vivo* have been reported. Using *in situ* hybridization, Quaglino et al. (1991) reported that a single injection of TGF-ß at the wound site significantly decreased expression of transin/stromelysin, a member of the neutral metalloproteinase family.

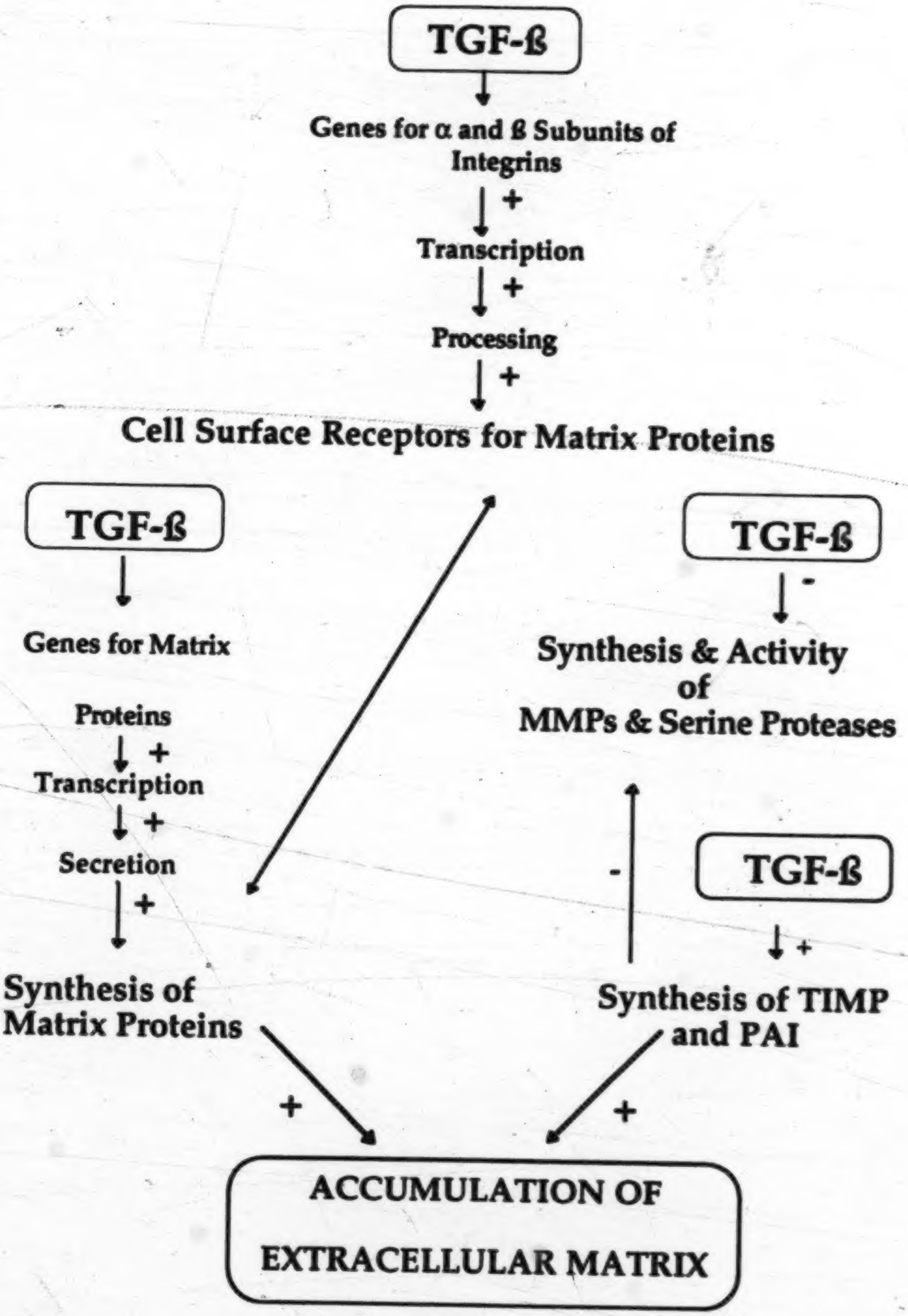
Cell Adhesion Receptors Cell adhesion receptors, integrins, are a family of heterodimeric membrane glycoproteins consisting of a large alpha subunit and a smaller beta subunit. The alpha subunit confers ligand specificity and the beta subunit seems to be common to different cell adhesion receptors. Integrins link the ECM and the cytoskeletal elements of the cell through small cytoplasmic domains which interact with actin filament connecting proteins. A large amount of evidence indicates that the ß1 integrin or VLA (very late antigen) family play important roles in cell adhesion, cell migration, cell proliferation and differentiation (Ruoslahti & Pierschbacher, 1987). At least four of the six members of this family, α2ß1, α3ß1, α5ß1, α6ß1, mediate the adhesion of cells to ECM proteins.

In 1989 Ignotz and collaborators reported that TGF-ß stimulates expression of cell adhesion receptors. They showed that treatment of a variety of cell types of both mammalian and avian species with TGF-ß promotes and increases adhesion to fibronectin and collagen substrates. Interestingly, thymocytes, less-adherent cells than fibroblasts or epithelial cells, also respond to TGF-ß with increased adherence to fibronectin substrate. Moreover, they showed that the induction of adhesion occurs at the level of mRNA, the rate of processing and translocation to the plasma membrane of the ß chain subunit. This group (Heino et al., 1989b) has also demonstrated that TGF-ß not only stimulates expression of ß1 integrins, but also regulates expression of members of the ß2 and ß3 families of integrins in a variety of fibroblastic cells. They showed that TGF-ß stimulates the expression of the vitronectin receptor (αvß3 integrin) in all tested fibroblastic cells. Vitronectin is a plasma glycoprotein that is important for the attachment and spreading of cells in culture and presumably *in vivo*. Since the kinetics of the vitronectin receptor and ß1 integrins were similar in response to TGF-ß, they suggested the presence of common TGF-ß-induced regulatory mechanisms in the expression of ß1 and ß3 integrins. Some evidence suggests that TGF-ß also modifies the adhesive behavior of certain tumor cells by altering the repertoire of cell adhesion receptors that they express. For example, Heino and

Massague (1989a) reported that TGF-ß induces a marked decrease in the adhesion of MG-63 human osteosarcoma cells to laminin-coated surfaces.

TGF-ß regulates not only the expression of integrins, but also modulates the expression of growth factor receptors. The stimulatory effect of TGF-ß on the expression of the EGF receptor has been reported (Assoian et al., 1984). Taken together, these data suggest that TGF-ß may control cell migration, differentiation and specific cell phenotype not only through regulation of the ECM, but also by specifically modulating the ability of the cell to adhere to different components of the ECM.

The schematic diagram on the next page shows a summary of the mechanisms involved in the positive effects of TGF-ß on the accumulation of ECM components and their interactions with cells.


TGF-ß
Genes for α and ß Subunits of Integrins
+
Transcription
+
Processing
+
Cell Surface Receptors for Matrix Proteins
TGF-ß
Genes for Matrix
Proteins
+
Transcription
+
Secretion
+
Synthesis of Matrix Proteins
TGF-ß
-
Synthesis & Activity of MMPs & Serine Proteases
-
TGF-ß
+
Synthesis of TIMP and PAI
+
+
ACCUMULATION OF EXTRACELLULAR MATRIX

Wound Healing The stimulatory effects of TGF-ß on ECM deposition and granulation tissue formation are well documented. Moreover, TGF-ß affects other fundamental events of the tissue repair process such as cell migration, proliferation, angiogenesis and remodeling.

Using the wound healing model of Hunt et al. (1967), Sporn and co-workers (1983) demonstrated that injection of TGF-ß1 into a wound chamber implanted subcutaneously in the back of the rat, accelerated the accumulation of total protein, collagen and DNA. In another study Roberts et al. (1986) showed that subcutaneous injection of nanogram quantities of TGF-ß1 into the nape of the neck of newborn mice, rapidly induced formation of a localized nodule of granulation tissue. This granulation tissue was characterized by an influx of inflammatory cells, fibroblast proliferation, synthesis of matrix proteins and neovascularization. Since the histology of sections removed from animals which had been injected with TGF-ß1 for 3 days and then left untreated for an additional 5 days, was the same as that of control sections, they concluded that these TGF-ß1-induced events are reversible. The ability of TGF-ß1 to correct the wound healing deficit in a wound chamber model has also been studied. Lawrence et al. (1986) showed that a combination of growth factors (EGF, PDGF, TGF-ß) are required to completely reverse the inhibition of wound repair induced by adriamycin. Interestingly, TGF-ß1 at certain concentrations was effective as an individual factor while the other factors were ineffective alone.

Evidence provided by Cromack et al. (1987) confirms that TGF-ß is an intrinsic component of wound fluid. Using aspirated wound fluid from Schilling-Hunt wire-mesh chambers implanted into the back of rats, they showed a time-dependent increase in the concentration of TGF-ß in wound fluid. Initial acute release of TGF-ß1 from platelets at the time of injury is believed to be an initiator for the cascade of events necessary for tissue repair. One of the first events in wound healing is the movement of inflammatory cells through the endothelial junctions in response to the chemotactic factors released in injured tissue. All components of the cellular infiltrate during the first stage of wound repair are known to be either a major source of TGF-ß (platelets) or to release a large amount of TGF-ß upon activation (macrophages and lymphocytes). TGF-ß is known as a potent chemotactic factor. It has been shown that this growth factor at femtomolar concentrations induces monocyte chemotaxis and at higher concentrations, it activates monocytes to secrete factors, particularly IL-1, which are mitogenic for fibroblasts (Schmidt et al., 1982). TGF-ß is also a potent chemoattractant for fibroblasts and may cause fibroblasts to migrate into the wound healing area (Wahl et al., 1987). Moreover TGF-ß may directly or indirectly, through the induction of PDGF, stimulate fibroblast proliferation in the wound site. In addition, the production of fibronectin, collagen and some species of glycosaminoglycans by fibroblasts is directly stimulated by TGF-ß. Also, as mentioned, TGF-ß enhances protease inhibitor synthesis and suppresses proteolytic enzymes. The outcome of these differential effects on the synthesis and degradation of matrix proteins is the stabilization of the newly formed matrix.

Although the production of transgenic mice over-expressing TGF-ß1 by Roberts's group resulted in embryonic lethality (Sporn and Roberts, 1992), the successful generation of transgenic mice that express active TGF-ß1 in the epidermis and transgenic TGF-ß-deficient mice have provided more direct evidence for the role of TGF-ß1 in skin development and wound healing (Sellheyer et al., 1993; Letterio and Roberts, 1996). This group has shown that the over-expression of the active form of TGF-ß1 in epidermis had a profound inhibitory effect on the epithelia. Based on histopathological and histochemical examination, they reported several major abnormalities in the transgenic skin: 1) fewer hair follicles, 2) thinner epidermis, 3) very compact orthohyperkeratosis, 4) growth arrest of epidermis and hair follicles. Interestingly, no changes have been reported in the histology of the dermis, collagen production, fibroblastic cell organelles, or collagen bundles or bands, suggesting that the over-expression of active TGF-ß1 in this model is restricted to epidermis and does not have side effects in the dermis. Very recently, the same group have successfully generated transgenic TGF-ß1-deficient mice (Letterio and Roberts, 1996). Based on the evaluation of the available data on the effects of exogenous TGF-ß1 on wound healing, it was expected that wound healing would be impaired in these animals. Surprisingly, they did not observe any difference in the initial rates of healing of excisional wounds between wild-type and the TGF-ß1-deficient mice. Since immunohistochemical examination of the wounds for TGF-ß1 in transgenic animals was positive, they suggested a maternal source for the TGF-ß1 protein in transgenic animals and its possible contribution to the healing process. To eliminate the effects of the maternally transferred TGF-ß1 protein, they reexamined the rate of excisional wound healing in TGF-ß1-deficient mice after depletion of the maternally transferred TGF-ß1 protein. Once again, they found that the healing was not impaired in these animals. In fact, when they evaluated the pathophysiology of wound healing in this model they found that not only was the rate of wound healing in the transgenic mouse normal but also that the wound healed with better quality (less scar). Moreover, they have reported an overexpression of the TGF-ß3 isoform in the granulation tissue of wounds in the transgenic animals which may be responsible for the improved quality of healing. It has been shown that the addition of neutralizing antibodies to TGF-ß1 and TGF-ß2 or exogenous TGF-ß3 to incisional wound in animals reduces scarring (Shah et al., 1995). These findings suggest that the *in vivo* expression of TGF-ß isoforms are interrelated. Moreover, these data suggest that caution should be exercised before conclusions are drawn about the role of TGF-ß in wound healing and remodeling. Certainly, generation of TGF-ß2- and TGF-ß3 transgenic mice, both deficient and over-expressing, will shed new light on the regulation and the *in vivo* activities of these multifunctional growth factors.

C. CYTOSKELETON AND GENE EXPRESSION

It is becoming increasingly apparent that cell morphology/organization of the cytoskeleton plays a significant role in signal transduction and regulation of gene expression (Jones et al., 1993). The organization of cytoskeletal proteins, particularly actin microfilaments, controls cell morphology. Extracellular matrix is known to determine the organization of the cytoskeletal network. Cells interact with the ECM through the transmembrane receptor "integrins" which integrate the ECM with the cytoskeleton. The interactions between the cytoskeleton and the intracellular domains of integrins are mediated through structural proteins such as vinculin, talin, α-actinin and paxillin, collectively forming focal adhesion plaques (Turner et al., 1990). The adhesion plaques are found to be associated with proteins such as tyrosine kinases, protein kinase C, Ca^{2+}-dependent proteases and protooncogene products which all are involved in signal transduction cascades (Burridge and Fath, 1989). Alterations in the organization of adhesion plaques (as a result of re-organization of ECM or cytoskeleton) can activate these regulatory molecules (Burridge and Fath, 1989). In this view, the cytoskeleton can be part of an information transmission system extending from the ECM, through the cytoplasm, and terminating at specific sites in the nucleus. The proposed interactions between the cytoskeleton, adhesion plaques, and regulatory proteins involved in the signal transduction cascade is illustrated in Figure I-4. The system can be activated by: a) humoral factors such as hormones, cytokines and growth factors, b) alterations in ECM adhesivity which can be profoundly affected by ECM components and the cell surface receptor integrins, and c) disorganization of cytoskeleton microfilaments caused by transient changes in the activity of intracellular capping proteins which regulate the addition of actin monomers to the ends of the actin filaments (cytochalasin-like activity).

D. PROGRAMMED CELL DEATH (APOPTOSIS)

Programmed cell death or "apoptosis" is a crucial process during embryogenesis and an important mechanism for the homeostasis of every higher organism (Ucker, 1991). The maintenance of normal cell number in the body is controlled by balancing cell proliferation with cell death. It has been suggested that tumor cells achieve non-stop proliferation by blocking the apoptosis pathway (Reed, 1994). The role of protooncogenes/oncogenes in apoptosis is well-documented (Collins and Rivas, 1993; Haecker and Vaux, 1994) and a number of cytokines including TGF-ß have been shown to be involved by the modulation of the expression of these genes. Nass et al. (1996) have shown that the apoptotic activity of TGF-ß is mediated through the inhibition of Bcl-xL in c-myc overexpressing mammary epithelial cells. TGF-ß has been shown to regulate apoptosis in epithelial, endothelial and immune cells, and most investigators have used these cell types to study the mechanisms involved in the survival of mammalian cells. However, Bauer and colleagues have investigated apoptosis in fibroblastic cells (Hofler et al., 1993). They have shown that the addition of exogenous TGF-ß to a co-culture of normal and transformed fibroblasts leads to a dramatic reduction of colony-forming transformed fibroblasts. They have also shown that the elimination of transformed fibroblasts by their normal counterparts is mediated by induction of apoptosis

(Jurgensmeier et al., 1994). Moreover, they have reported that in the presence of the optimum concentration of exogenous TGF-ß, the eliminative capacity of normal fibroblasts is the property of all cells within the population, but in the absence of exogenous TGF-ß, a minority of clones have eliminative activity which is a stable characteristic. Interestingly, they have found that with increasing passage number, more cell clones exhibit eliminative capacity in a suboptimal concentration of exogenous TGF-ß (Picht et al., 1995). Very recently, the same group (Langer et al., 1996) have reported that reactive oxygen species play a functional role in this phenomenon. However, the exact mechanisms involved in the TGF-ß-induced elimination of transformed fibroblasts by their normal counterparts have not been addressed. These findings may be important in better understanding of the elimination of myofibroblasts in the granulation tissue during wound healing and remodeling.

The interactions between cells and ECM components have been suggested to play a crucial role in the survival of epithelial and endothelial cells. Several lines of study have shown that normal epithelial cells undergo apoptosis when detached from substrate with RGD peptides or by growing the cells in suspension. Frisch and Francis (1994) have called this phenomenon *"anoikis"*. In Greek the word means "homelessness". The anoikis theory suggests that if a differentiated epithelial cell was appropriately in contact with a suitable matrix it survives, but loss of the contact leads to apoptosis. In an *in vitro* system, Streuli and colleague (1993) have investigated the effect of the interactions of mammary epithelial cells with ECM and showed that TGF-ß plays a role in the differentiation, growth and survival of these cells. Although the anoikis theory has not been directly examined, the results from the investigation suggest that cell-ECM interactions, epithelial cell survival and the expression of TGF-ß are interrelated. Perhaps, one of the best *in vivo* example of anoikis is the normal process of epidermal maturation, whereby the epithelial cells that are in touch with the basement membrane continue to grow, while those cells that lose contact and move away die. The expression of TGF-ß1 by suprabasal keratinocytes but not basal keratinocytes supports the idea that TGF-ß1 may play a key role in anoikis and apoptosis (Levine et al., 1993). Although, to date, the role of anoikis in apoptosis has been shown to be limited to epithelial and endothelial cells, a similar mechanism could be proposed to cause apoptosis in fibroblasts.

E. ABNORMAL WOUND HEALING: *Possible etiology of HSc*

Hypertrophic scars (HSc) are fibrotic skin disorders characterized by imbalanced deposition of ECM and disorganization of structural proteins in the dermis following injury. Although the etiology of these abnormal scars is unknown, several factors such as race, age, anatomical site of the wound, infection and type of the injury have been implicated in HSc formation.

A large body of evidence shows that the physicochemical properties of HSc are significantly different from those of normal dermis and mature scar. Knapp and co-workers (1977) reported that collagen fibers and fiber bundles in HSc are less organized and less cross linked than those in normal dermis and mature scar. Other investigators (Kischer et al., 1981; 1984) have shown that fibrillar collagen in HSc is not less organized but presents a particular organization and orientation. Both light and electron microscopic examination of these pathologic scars have revealed that they contain unique structural units of collagen referred to as "nodules" in which collagen fibers are oriented parallel to the skin surface in a "cigar-shaped" fashion (Kischer 1981). Moreover, it has been shown that collagen fibrils in the nodular region are narrower and more irregular than those in normal dermis and mature scar (Kischer et al., 1981; 1984). Bailey et al. (1975) have suggested that a time-dependent change in the profile of the fibrillar collagen cross-links in normal human scar tissue is similar to that in early post-natal growth followed by normal maturation. Retaining the characteristic of embryonic collagen profile, HSc escapes from this maturation process. Based on the weight of fractional salt precipitates, Bailey et al. (1975) reported a ratio of type III to I collagen of 1:2 in HSc which is higher than that of normal dermis (1:3.5), but not as high as in embryonic human skin (1:1). This group has also suggested that myofibroblasts (immature or transformed fibroblasts) are responsible for the overproduction of type III collagen in HSc. Significant alterations in glycosaminoglycans (GAG) including chondroitin sulfate, hyaluronic acid and dermatan sulfate in HSc have been reported. Shetlar et al. (1971) reported that concentration of hyaluronic acid in HSc is significantly lower than that in normal skin. Moreover, Alexander and Donoff (1980) have shown that dermatan sulfate is exclusively associated with the non-nodular region in HSc. Elevated levels of chondroitin-4-sulfate and low levels of dermatan sulfate in granulation tissue and HSc have been also reported (Shetlar et al., 1971). Since GAGs and large proteoglycans have a profound effect on tissue hydration, a significant increase in the water content of HSc (Bailey et al 1975) can be attributed to the improper expression of GAGs and proteoglycans. Moreover, it is believed that the relationship between GAGs and collagen plays an important role in scar architecture since the quantity and quality of collagen cross-links and composition of extracellular matrix GAGs are related. Recently, Scott et al. (1996) have quantified and characterized the proteoglycans in human post-burn hypertrophic and mature scars. They reported that the small dermatan sulfate proteoglycan, decorin, was lower and the large chondroitin sulfate proteoglycan, versican, and biglycan were markedly higher in HSc than in normal dermis or mature scar tissue. This group suggested that the abnormal morphology and disorganization of collagen fibrils in HSc is a consequence of reduction in decorin content in the tissue. Since large proteoglycans increase the water holding capacity of tissue, the abnormal expression of these proteoglycans in HSc supports the observation that HSc is over-hydrated. This characteristic influences the physical properties of the ECM and could be responsible at least in part for the inelastic quality of HSc tissue, placing the fibroblasts under considerable mechanical stress. It has been reported that TGF-ß increases versican and biglycan mRNA but decreases decorin mRNA in cultured fibroblasts (Kahari et al., 1991). The overexpression of versican and biglycan and underexpression of decorin in HSc may be a consequence of overexpression of TGF-ß, perhaps produced by activated fibroblasts, in the tissue.

In regard to the cell types that inhabit HSc, Kischer (1984) showed that the major cell type in HSc is an activated fibroblast while in mature scar and normal dermis the majority of fibroblasts are quiescent. Fibroblasts are the main cellular elements responsible for the homeostasis of the dermis. Therefore, alterations in their activity result in abnormal accumulation and/or degradation of matrix components. Mast cells are another cellular element of HSc. Statistical analysis of cell counts in granulation tissue, HSc and normal scars from deep thermal injuries has shown that HSc contains more mast cells than the other tissues examined (Kischer et al., 1978). Mast cell vesicles contain a substantial amount of the sulfated proteoglycan, heparin. Of significance, MacCaffrey et al. (1989) reported that the active form of TGF-ß can be released by heparin from inactive α2-macroglobulin-TGF-ß complexes. It is possible that mast cells intensify the *in vivo* biological activity of TGF-ß by this mechanism.

The dynamic events which may lead to the development of HSc have been studied. Kischer et al. (1990) proposed that granulation tissue carries the template for production of the nodules and HSc. They examined the morphology of the nodules and microvessels of fifty-six pieces of human granulation tissues from full thickness wounds implanted into subcutaneous pockets of nude mice (athymic mice) for 40 days. They reported that lateral branched microvessels and activated fibroblasts of the granulation tissue were essential for the nodular structure and scar formation since granulation of an earlier age, before lateral vascular branching, which also lacked activated fibroblasts, did not form nodules. Granulation tissue is known as a contractile structure that undergoes a massive reduction in cellularity during maturation and remodeling (Clark, 1993). It has been also suggested that the inhibition of the elimination of activated fibroblasts in granulation tissue is responsible for the development of HSc. The elimination of fibroblasts has also been shown during the resolution of HSc which is associated with a reduction in cellularity. A programmed cell death, apoptosis, has shown to be involved in the elimination process (Desmouliere and Gabbiani, 1996).

Based on the light and electron microscopic examination of a large number of HSc and keloid tissues, Kischer and colleagues suggested that hypoxia is an integral factor in the generation of pathological scars (Kischer et al., 1982a; 1982b; 1990). They have shown that the vast majority of microvessels in HSc are occluded and the occlusion persists throughout the period of hypertrophy. They believe that resolution of the occlusion corrects the oxygen tension and removes the stimulus for fibroblasts, finally, resulting in scar maturation (Kischer et al., 1990). The effects of oxygen tension and lactate on the *in vitro* activation of fibroblasts support this idea. It has been shown that fibroblasts not only survive in hypoxia but also proliferate and synthesize collagen in excess (Hunt et al., 1978). Moreover, it has been reported that lactate increases prolyl hydroxylase activity in cultured fibroblasts. This effect of lactate has been reported to be specific, since other cell metabolites did not show such an effect (Comstock & Udenfriend 1970). Lactate was considered as a prime candidate for stimulation of collagen synthesis by

Levene and Bates (1979). Interestingly, Falanga et al. (1991) have reported that hypoxia also upregulated the synthesis and secretion of TGF-ß by human dermal fibroblasts. The latent form of TGF-ß can be locally activated. Once activated, besides inducing other biological activity, TGF-ß can positively regulate its own expression through an autocrine mechanism (Obberghen-Schilling et al., 1988) which in turn propagates and perpetuates the activation of fibroblasts. Elevated levels of TGF-ß in various fibroproliferative disorders including HSc (reviewed by Tredget, 1994) and suppression of fibrotic activity by neutralizing the *in vivo* activity of TGF-ß (Border et al., 1990) provide further evidence for the involvement of TGF-ß in the pathogenesis of HSc.

In contrast to its stimulatory effects on fibroblasts, TGF-ß is known to inhibit growth and to induce apoptosis in endothelial cells (Tsukada et al., 1995). Thus, one can suggest that TGF-ß can play an equally important role in the development as well as the resolution of HSc. However, Scott et al. (1995) have reported a higher level of immunoreactive TGF-ß and decorin in mature scar compared to HSc and suggested that decorin may play a role in the resolution of the scars by acting as a "sink" for TGF-ß which is a potent fibrogenic factor.

F. THESIS AIMS

Available data on the pathogenesis of hypertrophic scars, a fibroproliferative disorder that frequently develops as a result of deep dermal injury, suggest a failure in extracellular matrix (ECM) metabolism. The scars are characterized by an altered composition and organization of the ECM and the presence of activated fibroblasts with altered morphology. However, the correlation between these alterations is not clear. I hypothesized that during wound healing and remodeling, the physicochemical properties of ECM undergo a series of changes which affect the morphology of fibroblasts and consequently modulate their activity. Since animal cells respond to changes in cell shape by altering gene expression, I investigated the effects of alterations in cell morphology on the expression of the genes for TGF-ß, a regulator of ECM, and proteins involved in ECM metabolism in dermal fibroblasts.

This thesis documents cell morphology and physicochemical properties of matrix as potent modulators of TGF-ß and collagenase gene expression, and growth and survival of dermal fibroblasts. These are factors which all play significant roles in ECM metabolism.

The specific aims of the study were:

I. To induce alterations in the morphology of fibroblasts grown on plastic (a rigid matrix) by chemical agents which disrupt the cell-matrix interactions/cytoskeleton by different mechanisms,

II. To examine the organization of the cytoskeleton microfilaments after morphological modification,

III. To evaluate the expression of TGF-ß1 at the level of transcription, mRNA and protein after morphological modifications,

IV. To evaluate expression of the genes for the proteins involved in ECM metabolism,

An *in vitro* model of fibroblast culture in a biologic matrix, type I collagen (CM), was developed to examine the hypothesis in an environment which mimics the *in vivo* situation better than monolayer culture on plastic. In addition, the model allowed the examination of the effects of re-organization of the collagen matrix and stress-relaxation of fibroblasts on gene expression, growth, and survival of dermal fibroblasts.

The specific aims were:

I. To induce alterations in the morphology of fibroblasts grown in CM by mechanical force generated during contraction of the matrix and stress-relaxation,

II. To examine the morphology and organization of the cytoskeleton microfilaments before and after contraction of the matrix and stress-relaxation,

III. To evaluate the expression of TGF-ß at the level of the mRNA and protein in CMs and monolayer cultures,

IV. To evaluate expression of the mRNA for proteins involved in ECM metabolism in CMs and monolayer cultures,

V. To examine the effects of the nature and re-organization of the matrix on cell growth and apoptosis.

Cytoskeleton of the fibroblasts grown on plastic was disrupted by three different chemical agents, trypsin, EGTA, or cytochalasin C (Chs) as described in Chapters II and III. Trypsin and EGTA interrupt the cell-matrix interactions by proteolysis and chelation of Ca^{2+} respectively. Cytochalasin specifically binds to the fast-growing end (plus end) of filamentous actin in the cell and shifts the kinetics of the assembly of microfilaments towards dissociation, resulting in inhibition of filamentous actin and stress-fiber formation. Disruption of cytoskeleton by these chemicals are conventional and widely-used approaches to study the correlation between cell morphology and cell function (Rees et al., 1977; Aggeler et al., 1990; Benya and Padilla, 1993; Higgins et al., 1995). However, these agents may have effects on the cells other than disruption of cell-matrix interactions and cytoskeleton. In this work, their common effects on the cytoskeleton and cell morphology are of interest and the undesired effects, wherever possible, are avoided. For example, it has been shown that Chs at a concentration of 4 µg/ml and above partially inhibits protein synthesis (Cooper, 1987). This possible undesired effect, may be avoided by using a lower concentration of Chs (1-2 µg/ml) which was adequate to disrupt the cytoskeleton microfilaments of dermal fibroblasts. Cells treated with EGTA or trypsin were washed in DMEM, seeded for 4-6 h and allowed to correct the imbalanced concentration of cations and some of the cell-surface components which may be lost as a result of the treatments.

It is the aim of Chapter II to first examine the effects of re-organization of the cytoskeleton on the expression of TGF-ß1 mRNA and protein and the levels of the mRNA for proteins involved in ECM metabolism. The second aim of Chapter II is to examine whether fibroblasts derived from normal dermis and HSc tissue respond differently to the re-organization of the cytoskeleton.

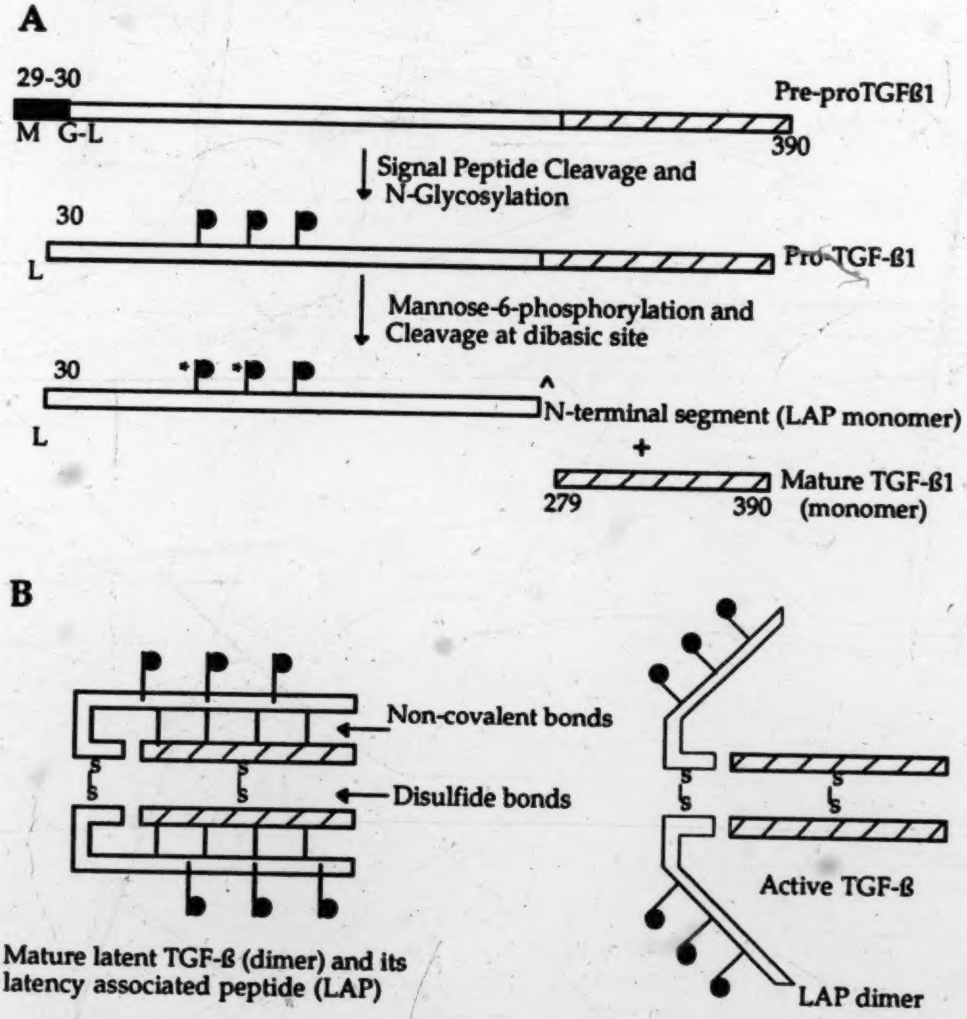
The aim of Chapter III is first to evaluate the effects of re-organization of cytoskeleton on the transcription of the genes of interest. The second aim of Chapter III is to briefly investigate the mechanisms which may be involved in the cell shape-related induction of TGF-ß1. The activation of the TGF-ß1 gene is believed to be largely governed by several AP-1 elements located in the promoters. (Kim et al., 1989a). To examine the possible role of these elements, the levels of the mRNA for the AP-1 complex components, *c-fos* and *c-jun* oncoproteins, were evaluated. When the levels of the mRNA for these oncoproteins were to be evaluated, the cytoskeleton was disrupted by trypsin. Trypsin was chosen over EGTA or Chs because: 1) cells harvested by trypsin and replated for a short time exhibit a similar cell shape and organization of the cytoskeleton microfilaments to those of cells treated with EGTA as described in Chapter II. Moreover, a large number of cells are required for these experiments and the yield of cells from confluent cultures using trypsin is much greater, and, 2) induction of *c-fos* mRNA and protein following disruption of the cytoskeleton with Chs in human fibroblasts has been already investigated (Zambetti et al., 1991). Likewise, when the role of inhibition of protein synthesis was to be examined, Chs was chosen over EGTA or trypsin because in the presence of cycloheximide (Cxm), trypsinized or EGTA treated cells do not adhere effectively and thus there were insufficient number of cells for extraction of RNA.

Type I collagen matrix (CM) is permissive for changes in the morphology of the embedded fibroblasts when the matrix is allowed to contract (Bell et al., 1979). The basis for the changes is believed to be the mechanical force which is generated by re-organization of the collagen fibrils and/or stress- relaxation of fibroblasts. The aim of Chapter IV is first to define and compare the morphology of the fibroblasts grown in anchored (ACM), stress-relaxed (St.rCM) and floating (FCM) matrices. These types of *in vitro* fibroblast culture permit the investigation of the correlation between the morphology and activity of fibroblasts in an environment which has at least two advantages over the monolayer cultures on plastic. First, collagen, mimics the *in vivo* situation better than plastic. Second, the alterations in cell morphology do not need to be induced by chemical agents which may have undesired side effects. Moreover, the effects of re-organization of the fibrillar collagen and rigidity of the matrix on fibroblast behavior can be investigated in these models. Of particular interest in relation to wound healing and HSc is the role of rigidity and re-organization (remodeling) of the matrix on the activation of fibroblasts. It is the second aim of Chapter IV to compare the levels of expression of the mRNA for TGF-ß1, collagenase and procollagen I (α1) in the cells grown in CM (flexible matrix) and plastic (rigid matrix). The growth activity (proliferation) and survival (apoptosis) of fibroblasts were also compared and the results discussed with respect to the relevant *in vivo* findings in the literature on the pathophysiology of wound healing.

In Chapter V, (General Discussion and Conclusions), the results are generally discussed and conclusions are drawn in relation to the activation of fibroblasts during cutaneous wound healing, remodeling, and development and resolution of HSc.

Figure I-1:

Proposed post-translational processing and structure of latent TGF-ß1. A) Signal peptide (solid box) and its cleavage site at the Gly-Leu peptide linkage, N-linked oligosaccharide (▶). Mannose-6-phosphate residues (*). Proteolytic cleavage at the dibasic cleavage site (^). Mature TGF-ß1 monomer (cross-hatched box) is separated from the amino - terminal glycopeptide (open box), latency associated peptide (LAP). B) Disulfide bond formation and homo-dimerization. The TGF-ß1 remains non-covalently bound to LAP dimer (latent TGF-ß1) until it becomes activated.



Mature latent TGF-ß (dimer) and its latency associated peptide (LAP)

Figure I-2:

Schematic diagram showing human TGF-ß1 gene and the mRNA. A) The regulatory regions in the 5'-flanking region of the human TGF-ß1 gene. Position of enhancer-like element (E, open box), negative regulatory elements (N1 and N2, solid boxes) and positive regulatory element (P, cross-hatched box) are shown relative to the transcription start site (+1). B) Seven exons (E1-E7) and 6 introns (—) of the gene. The boxed-region of the mRNA represents the coding sequence for the TGF-ß1 precursor (390 amino acid) and the cross-hatched-region represents the coding region corresponding to the mature TGF-ß1 (112 amino acid which begins at amino acid 279 of the precursor)

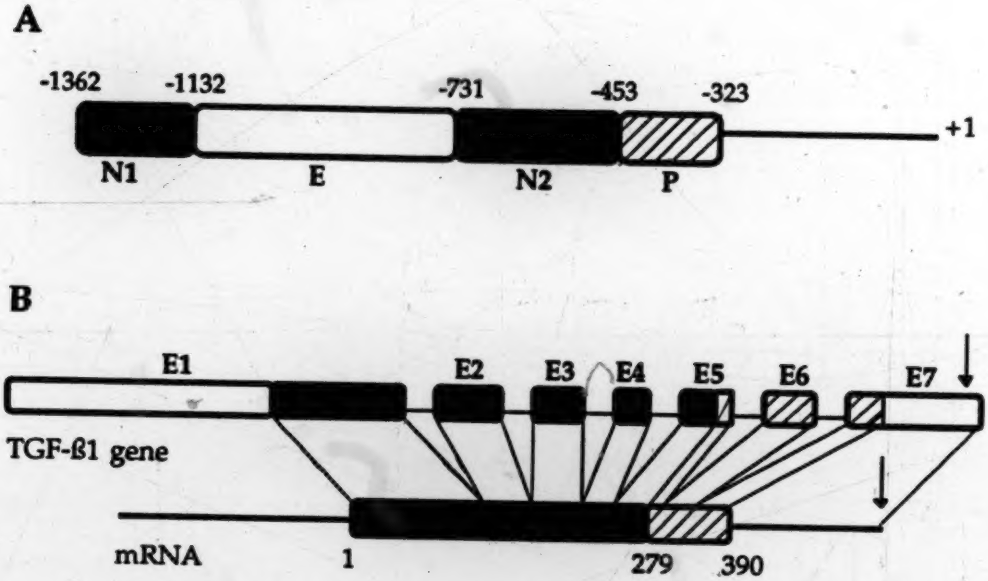


Figure I-3:

A) Schematic diagram showing the type I-V TGF-ß receptors and a TGF-ß binding trans-membrane protein, endoglin. The cross-hatched bar represents cell membrane (CM). The arrow shows the potential cleavage site and the balls and sticks (⫯) represent GAG chains of the type III receptor. B) The initiation of signaling following binding of active TGF-ß ligand to type II receptor and trans-activation of type I receptor. The rectangular solid boxes and the ¯Ps show phosphorylated residues of type II and I receptors.

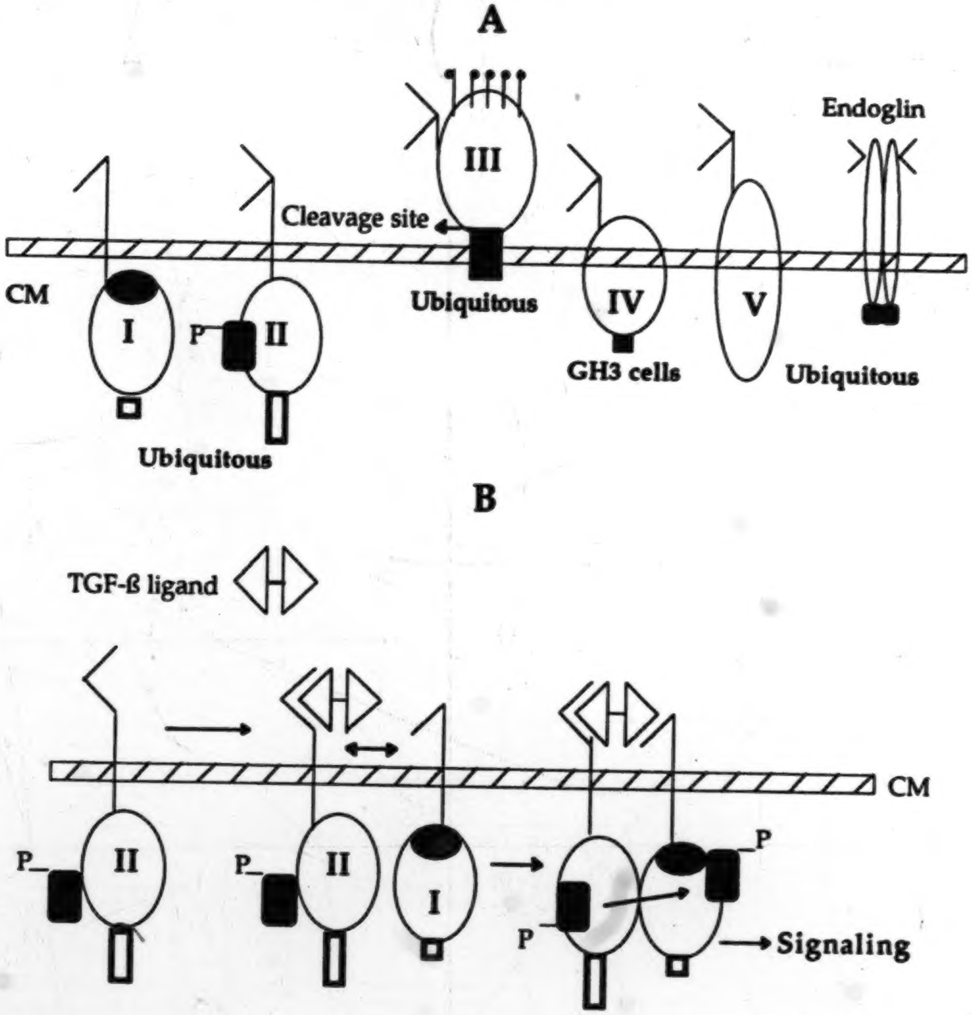
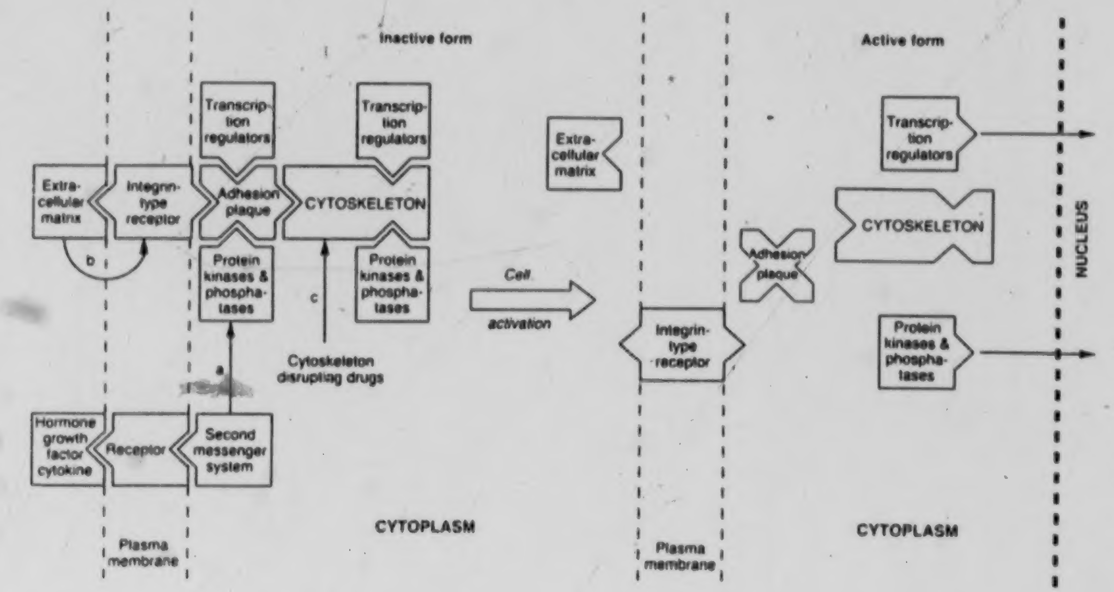


Figure I-4:

Interaction between the cytoskeleton and the signal transduction system, and the compartmentation model of regulatory molecules involving the cytoskeleton to control signal transduction to the nucleus. a) Cellular stimulation by humoral factors. b) Alterations in the ECM adhesivity or the cell surface matrix receptors. c) Transient disorganization of the cytoskeleton microfilaments (changes in intracellular cytochalasin-like activity). The diagram is taken from Ben-Ze'ev, BioEssays, vol. 13, No. 5, 1991.



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II

ALTERATION IN CELL MORPHOLOGY TRIGGERS EXPRESSION OF TRANSFORMING GROWTH FACTOR- ß1, COLLAGENASE AND TIMP-I EXPRESSION IN DERMAL FIBROBLASTS *

INTRODUCTION

In recent years, it has become apparent that cytokines, including transforming growth factor-beta (TGF-ß), play a crucial role in wound healing and tissue remodeling. TGF-ß1 is a potent chemotactic factor for fibroblasts (Postlethwaite et al., 1987) and inflammatory cells (Wahl et al., 1987; Brandes et al., 1991). It also stimulates proliferation of immature fibroblasts (Moses et al., 1985) resulting in an increased fibroblast population, the main cellular source of extracellular matrix (ECM) components at the site of injury. TGF-ß1 stimulates the synthesis of major matrix components including collagens, fibronectin (Ignotz et al., 1987) and proteoglycans (Bassols et al., 1988). It also stimulates the production of proteinase inhibitors, including plasmin-

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ogen activator inhibitor (Laiho et al., 1986a) and tissue inhibitor of metalloproteinases (TIMP), and inhibits the production of matrix proteinases such as collagenase (Edwards et al., 1987) and plasminogen activator (Laiho et al., 1986b). Collectively, these biologic activities lead to the accumulation and stabilization of the nascent matrix which is vital to wound healing.

Although reparative functions of TGF-ß1 are beneficial to wound healing (Mustoe et al., 1991; Pierce et al., 1989), overactivity of this growth factor could be responsible for scarring and fibrosis. Overaccumulation of ECM, changes in ECM components and architecture and activated fibroblasts are common hallmarks of fibroproliferative disorders (FPDs). We have reported recently that TGF-ß1 is overexpressed in post-burn hypertrophic scars (HSc, Ghahary et al., 1993). Elevated levels of TGF-ß1 in various fibrotic conditions (Ghahary et al., 1993; Khalil et al., 1991; Czaja et al., 1989; Gruschwitz et al., 1990) and the suppression of fibrotic activity by neutralizing the activity of TGF-ß1 *in vivo* (Border et al., 1990) provide evidence for its involvement in the pathogenesis of FPDs.

Despite progress in defining the biological activities of TGF-ß in health and disease, the mechanisms which regulate TGF-ß synthesis are not yet clear. Several factors such as differentiation (Nilsen-Hamilton et al., 1990), oxygen tension (Falanga et al., 1991), cytokines including TGF-ß itself (Obberghen-Schilling et al., 1988) members of the steroid hormone superfamily (Wakefield et al., 1990) and ECM components (Streuli et al., 1993 may be involved in the regulation of TGF-ß expression. The ECM not only serves as a scaffold for cells but also controls cell shape and regulates many cellular functions by way of the cell cytoskeleton. The cytoskeleton undergoes remarkable changes during cell division, cell migration and differentiation, events fundamental to wound healing. Alterations in the cytoskeleton subsequently affect cell-matrix and cell-cell interactions, resulting in alteration in cellular phenotype and function. In this study I have examined the role of alteration in cytoskeleton in the expression of TGF-ß1 in dermal fibroblasts. Alterations in cytoskeleton were induced by trypsin, ethyleneglycol-bis(ß-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) or cytochalasin C (Chs).

MATERIALS AND METHODS

Cell Culture

Fibroblast cultures from normal skin and hypertrophic scar tissue were established according to Nakano and Scott (1986). Briefly, punch biopsies were collected into Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and minced into small pieces of less than 0.5 mm in dimensions. The tissue fragments were washed six times with sterile medium, and distributed into 60 x 15 mm petri dishes, four pieces per dish. DMEM (3 ml) with 10% (v/v) FBS was added to each dish and incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every 5 days. After 4 weeks, the cells, predominantly fibroblasts, were harvested from the dishes by a brief (3-5 min) treatment with 3 ml of 0.1% (w/v) trypsin (Sigma) and 0.02% (w/v) EDTA (Sigma) in PBS (pH 7.4) and transferred to 75 cm^2 culture flasks. At confluence, the cells were subcultured by splitting 1:5 by trypsinization. In this study I used paired hypertrophic scar (HSc) and normal (N) dermal fibroblast cultures from passage four to seven.

Antibodies and Probes

Two polyclonal anti-TGF-ß1 antibodies, anti-CC and anti-LC, and similarly prepared non-immune IgG were provided by Kathleen C. Flanders (National Cancer Institute, NIH, Bethesda, MD, USA). The specificity of these antibodies has been examined by western blot, radioimmunoassay, enzyme-linked immunosorbent assay and immunohistochemistry (Flanders et al., 1989). It is believed that anti-CC principally recognizes extracellular TGF-ß, while anti-LC reacts with intracellular TGF-ß. The complementary DNA (cDNA) probe for TGF-ß1 was characterized by Ardinger et al. (1988) and donated by Dr. G.I. Bell, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, Illinois, USA. The type I procollagen cDNA probe was characterized and provided by Drs. G. Tromp (1988), H. Kuivaniemi (1988) and L. Ala-Kokko (1989, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA, USA). The probes for human fibronectin (Bernard et al., 1985), TIMP (Durfy et al., 1986) and collagenase (Witham et al., 1986) were purchased from American Type Culture Collection (ATCC, 12301, Parklawn Drive, Rockville, MD, 20852, USA).

Disruption of Cytoskeletal Organization

Disruption in cytoskeletal organization was induced by either trypsin, EGTA (ethyleneglycol-bis (ß-amino ethyl ether) tetra acetic acid) (Sigma), or cytochalasin C (Sigma). Confluent cells were harvested from culture flasks using either 3 ml of 0.1% (w/v) trypsin and 0.02% (w/v) of EDTA in PBS (pH 7.4) for 3 min or 6 ml of 0.05% EGTA in Hanks' Balance Salt Solution (HBSS) for 45 min. Cells were washed twice with DMEM+10% (v/v) FBS, resuspended in the same media and replated by splitting 1:3. After 4 h, cells were used for total RNA extraction. To examine the time course of TGF-ß1 mRNA expression, the total RNA was extracted from trypsinized cells after replating for 4, 8, 12, 24 and 96 h (confluent culture). Cytochalasin C (Sigma) was first dissolved in 95% ethanol (1 mg/ml) and used in a final concentration of 1 μg/ml in DMEM+10% (v/v) FBS. Confluent cells were treated with Chs C for 4 h. At the end of this period cells were washed with PBS for 3 min and immediately used for RNA extraction. The viability of Chs treated cells was more than 90% as judged by trypan blue dye exclusion and by recovery of cells following replacement of the medium.

Immunocytochemistry

A rabbit peroxidase anti-peroxidase immunostaining kit (Zymed, So. San Francisco, CA, USA) was used to localize and detect TGF-ß1. Cells were seeded on glass coverslips (22 mm x 22 mm) and allowed to grow for 4 or 24 h in DMEM+10% (v/v) FBS. The coverslips were then removed from the medium and fixed in 4% (w/v) paraformaldehyde in PBS for 18 h at 4^{o} C. Cells fixed on coverslips were washed twice in PBS for 5 min each and endogenous peroxidase was blocked with 4% hydrogen peroxide (30%) in 50% (v/v) methanol solution. After permeabilization with 0.05% (v/v) of polyoxyethylene sorbitan (Tween 20) in PBS, the nonspecific binding was blocked with 10% normal goat serum. The coverslips were then treated with the primary antibodies, rabbit anti-CC (12.5 ng/ml) or anti-LC (40 ng/ml), for 18 h at 4^{o} C in a humidified chamber. The coverslips were then washed with PBS and treated with bridging antibody, goat anti-rabbit IgG, 1/250 dilution of stock according to the manufacturer's instructions. After washing with PBS, the coverslips were treated with peroxidase anti-peroxidase (PAP) complex according to the manufacturer's instructions. The coverslips were then washed in PBS and covered with a 3-3'diaminobenzidine solution (DAB) containing 0.03% hydrogen peroxide for 10-20 min. Stained coverslips were lightly counterstained with hematoxylin for 10 seconds, washed in running water for 3 min, and then dehydrated and mounted for microscopy. In negative controls, primary antibodies were replaced with the same concentrations of non-immune normal rabbit IgG.

Visualization of Cellular Actin Filaments

The organization of cytoskeleton actin filaments was visualized by staining with rhodamine-labeled phalloidin. Confluent cells were harvested either by trypsin or EGTA and replated for 4 or 24 h on sterile coverslips. Some of the 24 h cultures were treated with 1 µg/ml of Chs C in DMEM+10% (v/v) FBS for 4 h. The coverslips were then fixed in 4% (w/v) paraformaldehyde in PBS at room temperature for 3 h. Cells were then washed in PBS and treated with 1/200 dilution of stock solution of tetrarhodamine isothiocyanide-labeled phalloidin (50 µg/ml of 70% v/v ethanol, Sigma) for 15 min in the dark. After washing with PBS, they were mounted on glass slides with glycol/P-phenylenediamine for microscopy.

RNA Extraction and Northern Blot Analysis

Fibroblast cell layers were lysed and pooled in 6 ml of 4 M guanidinium isothiocyanate (GITC) solution and total RNA from each individual cell lysate was then isolated by the GITC/CsCl procedure of Chirgwin et al. (1979). Briefly, the RNA was extracted from the guanidinium isothiocyanate homogenate by ulteracentrifugation through a dense cushion of cesium chloride. Samples were centrifuged in a Beckman SW 50.1 rotor at 25,000 rpm for 12-16 h at 22° C. The RNA pellet were then dissolved in 1 ml of a warm (68° C) TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.0) and precipitated by adding 3 volume of cold (-20° C) ethanol and 0.1 volume of sodium acetate (3 M, pH 5.2). The solution was kept for at least 4 h at -20° C and the precipitate was collected by centrifugation at 6000 rpm for 20 min at 4° C. The precipitate was dissolved in TE buffer (pH 8.0) and the concentration was estimated by measuring the optical density (OD) of the solution at wavelength of 260 nm using a single-beam spectrophotometer (PU 860, UV/VIS/NIR, S&I, Philips, Pye Unicam Ltd, York Street, Cambridge CB1 2PX, England). An OD of 1 corresponds to approximately 40 µg/ml of RNA. Total RNA (8 µg) from each individual fibroblast culture was then separated by on a 1% (w/v) agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filters. To ensure that the gel lanes were loaded with similar amounts of total RNA, the quantity of 18S and 28S ribosomal RNA were compared visually by ethidium bromide fluorescence. The blots were baked for 2 h at 80° C under a vacuum and then prehybridized for 4 h at 42° C in a prehybridization solution. Hybridization was performed at 42° C in the same solution for 16-20 h using either human TGF-ß1, fibronectin, type I procollagen, collagenase or TIMP-I cDNA probes. The cDNA probes were labeled with ^{32}P-alphα–dCTP by nick-translation. The filters were initially washed at room temperature with 2x SSC and 0.1% SDS for 1 h and finally washed for 30 min at 65° C in 0.2x SSC and 0.1% (w/v) SDS. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at -70° C in the presence of an intensifying screen. The quantitative analysis of autoradiographs was accomplished by a transmission densitometer (E-C apparatus model EC910, Fisher Scientific). Since the expression of fibronectin mRNA is not altered by each treatment, loading of total RNA was also normalized by using the cDNA probe for fibronectin which corresponded well with the ethidium bromide method of controlling loading.

TGF-ß growth inhibition assay

The mink lung epithelial cell, Mv1Lu, (CC1-64, American tissue culture type) growth inhibition assay and preparation of conditioned media were conducted according to Danielpour et al. (1989). Briefly, subconfluent cultures were trypsinized, washed in assay media (DMEM, 0.2% v/v FBS, 10 mM HEPES and 1% v/v penicillin/streptomycin antibiotic, GIBCO), seeded at 5 x 10^5/0.5 ml per well in 24 well plates and incubated for 1 h at 37° C in an atmosphere of 10% CO_2. At the end of this incubation period, the serum-free conditioned medium (natural or acidified) or standard amount (serial dilution of 40 to 0.5 pM) of TGF-ß1 was added, and 22 hr later cells were pulsed with 0.25 µCi of ^{3}H-labeled thymidine per well for 2 h at 37° C. Cells were then fixed, washed and the radioactivity was measured in the cell lysate collected from each well. All samples were assayed in triplicate. The standard curve was obtained with dilutions (80 - 0.5 pM) of the TGF-ß standard to convert the inhibition of DNA synthesis into concentration of TGF-ß. The levels of TGF-ß in conditioned media were calculated using the corresponding standard curve.

Statistical Analysis

Differences in mRNA expression of different cell populations as measured by densitometry on Northern blots, were tested using Student's paired t-test, p values of <0.05 were considered significant.

RESULTS

Immunocytochemical Staining As shown in Figure II-1, normal fibroblasts seeded for 4 h showed intense staining with anti-CC (panel a) and anti-LC (panel c) antibodies specific for TGF-ß1. In contrast, cells seeded for 24 h exhibited little or no staining with these antibodies (panel b and d respectively). There is an apparent association between cell morphology and the expression of anti-CC and anti-LC reactive TGF-ß1 in dermal fibroblasts, since there was a remarkable difference in the morphology of cells seeded for 4 h and the morphology of cells seeded for 24 h. In addition, I observed that at the center of 24 h cultures, the cells which exhibited a bipolar shape and more cell-cell contact did not express anti-CC (panel b) and anti-LC (panel d) reactive TGF-ß1, while at the periphery of these cultures the cells which presented a polygonal shape and less cell-cell contact stained with both antibodies (panel e and f). Anti-CC antibody is known to detect principally extracellular TGF-ß1. However, cell-associated anti-CC reactive TGF-ß1 was found in this study. A similar observation was earlier reported by Kane et al. (1991) in the wounded epidermis 24 h after injury. The expression of anti-CC and anti-LC reactive TGF-ß1 in HSc cells in the above-mentioned conditions was similar to that of normal cells (data not shown).

Actin Filament Organization   Figure II-2 shows the organization of filamentous actin in normal fibroblasts visualized by rhodamine-labeled phalloidin, a fungal metabolite which reacts specifically with filamentous actin (Cooper, 1987). The cells harvested either by trypsin (panel a) or EGTA (panel b) and replated for 4 h were mainly respreading and displayed a rounded or polygonal shape with little cell-cell contact. The filamentous actin appeared to be undergoing assembly with undefined organization in respreading cells. In contrast, the cells seeded for 24 h (panel d) exhibited well-organized actin filaments in the form of bundles, known as stress fibers (Gabbiani et al., 1972). These cells exhibited a bipolar shape with a high level of cell-cell contact. Confluent cells treated with Chs C for 4 h (panel c) were morphologically different from 4 h-seeded and 24 h-seeded cells. These cells showed a perturbed cytoskeleton with actin filaments which were undergoing disassembly. Neither a defined pattern of organization of filamentous actin nor a particular cell morphology was displayed by these cells. Fibroblasts from HSc dermis exhibited similar cytoskeletal organization and cell morphology in the above mentioned conditions (data not shown).

Northern Analysis of TGF-ß1 mRNA   As shown in Figure II-3, trypsinized cells replated for 4 h (lanes H', N') showed a significant increase (268±45%, $n=4$, $p<0.05$) in TGF-ß1 mRNA (2.5 Kb) as compared to their confluent counterparts (lanes H, N). When the same blot was rehybridized with a cDNA for fibronectin, the trypsinized cells and confluent cells showed a similar level of fibronectin mRNA (7.8 Kb). To examine whether the increase in the expression of TGF-ß1 mRNA is due to disassembly/reassembly of cytoskeleton or is a trypsin-induced effect, cells were harvested using EGTA, a chelating agent. As shown in Figure II-4, EGTA-treated cells (panel A, lanes HE, NE) showed a significant increase (336±67%, n=4, $p<0.05$) in the expression of TGF-ß1 mRNA as compared to their confluent counterparts (lanes H, N). I observed similar results when I used Chs C which inhibits the assembly of actin microfilaments. Northern analysis of total mRNA extracted from the confluent cells treated with Chs C for 4 h (Figure II-4, panel A, lanes H_C, N_C) showed a significant increase (262±75%, n=4, $p<0.05$) in TGF-ß1 mRNA as compared to the control (Figure II-4, panel A, lanes H, N). The level of fibronectin mRNA remained unchanged in EGTA- and Chs C-treated cells as judged by hybridization of the same blot with a fibronectin cDNA (Figure II-4, panel A). The ethidium bromide staining of 28S and 18S ribosomal RNA (Figure II-3, 4, panel B) and fibronectin cDNA probe were used as loading control. The autoradiograms of 4 pairs of hypertrophic scar and normal fibroblast cell strains were individually quantitated using densitometry and the average TGF-ß1/fibronectin mRNA ratio for treated cells was compared to that of confluent cells (control). A significant increase in TGF-ß1 mRNA expression in both HSc and normal dermal fibroblasts was observed following disruption of cytoskeleton either by trypsin, EGTA or Chs C (Figure II-3, 4, panel C). Although both HSc and normal fibroblasts responded in a similar manner, the magnitude of the increase in TGF-ß1 mRNA was more pronounced in HSc fibroblasts following trypsin and EGTA treatments.

I have also examined the effect of the reorganization of the cytoskeleton on the time course of TGF-ß1 mRNA expression. As shown in Figure II-5, Northern blot analysis of total RNA from trypsinized cells plated for 4, 8, 12, 24 and 96 h (confluent culture) showed a significant increase in TGF-ß1 mRNA as early as 4 h, peaking at 12 h (compared to that in confluent cells) and significantly decreased by 24 h (compared to that in 12 h).

TGF-ß growth inhibition assay The level of TGF-ß protein secreted into the serum-free media by confluent and 24 h seeded cells was determined using TGF-ß mink lung epithelial cell growth inhibition assay. These results showed that almost all of the secreted TGF-ß was in the latent form. The level of the active form of TGF-ß in the media was undetectable (the sensitivity of the assay is in range of 0.05-0.1 pM). However, acidification of the conditioned media revealed that the concentration of TGF-ß was 4 times (38.5 pM/1.2×10^6 cell) greater in 24 h cultures than in confluent cultures.

Northern analysis of collagenase and TIMP-I mRNA Fibroblasts are the major cellular source of ECM proteins and enzymes involved in ECM metabolism. Figure II-6 shows the effects of disruption of the cytoskeleton on the expression of mRNA for type I procollagen, a major matrix protein, collagenase and TIMP-I, two important proteins involved in ECM degradation. As shown in Figure II-6, trypsinized cells replated for 4 h (lanes H', N') showed significant (384±80%, n=4, $p<0.05$ and 325±62%, n=4, $p<0.05$) increases in the expression of collagenase mRNA (2.1 Kb), and TIMP-I mRNA (0.7 Kb) respectively, as compared to their confluent counterparts (lanes H, N). Similar result were seen when cells were treated with EGTA or Chs C (Figure II-7). Cells harvested by EGTA and replated for 4 h showed significant increases in the expression of collagenase mRNA (238±39%, n=4, $p<0.05$) and TIMP-I mRNA (365±65%, n=4, $p<0.05$) as compared to their confluent counterparts. Confluent cells treated with Chs C for 4 h also expressed significant levels of collagenase mRNA (256±48%, n=4, $p<0.05$) and TIMP-I mRNA (257±35%, n=4, $p<0.05$) as compared to control. Although I found a variation in the expression of procollagen mRNA between cell strains, no significant change was observed in the expression of type I procollagen mRNAs in trypsinized cells (Figure II-

6), EGTA-treated cells or Chs C-treated cells (Figure II-7). These results suggest that the disruption of cytoskeleton also induces the expression of collagenase and TIMP-I mRNA in a selective fashion.

DISCUSSION

Although there has been considerable progress recently in the understanding of the intricate role of TGF-ß in physiological and pathological conditions, the mechanisms that govern the expression of these multifunctional growth factors are not completely understood. In this study I provide evidence to suggest that the disruption of the cell cytoskeleton alters mRNA expression for TGF-ß1, collagenase, and TIMP-I. I observed initially that cells replated after trypsinization showed a very rapid induction of TGF-ß1. Although disruption of the cytoskeleton is an immediate and obvious consequence of trypsinization, the up-regulation of TGF-ß1 following this treatment may be caused by the loss of some proteinase-susceptible cell surface component. Therefore, I tested the effects of EGTA and Chs and found that, while both caused a significant increase in TGF-ß1 mRNA, EGTA was more effective. The morphology of the cells subjected to these two treatments was quite different. The EGTA-treated cells were respreading and the microfilaments were undergoing assembly, while the Chs-treated cells were rounding up and the microfilaments were undergoing disassembly. Whether the difference in the magnitude of the effect is due to the state of the microfilaments or to the chelation of bivalent cations remains to be elucidated. Apparently, cytoskeletal status-induced TGF-ß1 mRNA is not a part of a general phenomenon since the expression of fibronectin and type (I) procollagen mRNA was not significantly affected by these treatments.

Evaluation of TGF-ß in fibroblast conditioned media revealed that the disruption of cytoskeleton increases the level of secreted TGF-ß protein. As do most cultured normal cells (Lyons et al., 1988), the dermal fibroblasts in these *in vitro* experiments also secreted TGF-ß in a latent form. Although I did not detect the active form of this growth factor, this does not necessarily imply that TGF-ß secreted by dermal fibroblasts *in vivo* is also inactive. Latent TGF-ß can be activated by acidification or exposure to proteases such as plasmin (Lyons et al., 1988) suggesting that the tissue microenvironment may regulate the activation of TGF-ß *in vivo* .

Fibroblasts regulate the turnover of ECM via the synthesis and secretion of matrix proteinases, matrix metalloproteinases (MMPs) and proteinase inhibitors (TIMPs). Up-regulation of collagenase and TIMP-I mRNA following the disruption of cytoskeleton reported here is unlikely to be related to an autocrine effect of TGF-ß since the TGF-ß released by these cells in culture is not biologically active. I suggest that the induction of collagenase and TIMP-I mRNA is also cell-shape related. The different treatments used in this study had similar inductive effects suggesting that the induction is not a consequence of trypsinization. Several studies have shown that changes in cell morphology induce expression of collagenase and stromelysin in cultured fibroblasts and this induction has been attributed to the reorganization of cytoskeletal elements (Bauer et al., 1982; Aggeler et al., 1990a; 1990b) although the mechanism(s) is not yet clear. Werb et al. (1989) provided evidence that the interaction of fibronectin with its integrin receptor may be involved. An effect of cell shape and cytoskeletal architecture on the expression of TIMP appears not to have been previously reported.

Recently, Streuli et al. (1993) showed that the absence of the basement membrane specifically up-regulates the expression of TGF-ß1 in mammary epithelial cells. Interactions between cells and matrix are mediated by membrane spanning cell-surface adhesion receptors, known as integrins. The cytoplasmic domains of integrins interact with actin filament-connecting proteins such as vinculin, talin and α-actinin, providing the linkage between the ECM and cytoskeleton. I believe that the absence of ECM may be associated with alterations in cell morphology and re-organization of the cytoskeleton which could then be a trigger for the induction of several genes, including TGF-ß1.

CONCLUSION

This study shows that re-organization of cytoskeleton stimulates dermal fibroblasts to express TGF-ß1 mRNA and protein. The overexpression of TGF-ß1 has been reported in HSc tissue (Ghahary et al., 1993). However, in the present study a similar response to the expression of TGF-ß1 was seen in fibroblasts derived from normal dermis and HSc. Thus, the *in vivo* overexpression of TGF-ß1 in HSc tissue is probably either due to local factors and influences or the production of this growth factor by another cell population. Induction of collagenase and TIMP-I mRNA but not fibronectin or type I collagen mRNA following alterations in cell morphology suggest that the re-organization of cytoskeleton differentially modulates the gene expression of proteins involved in ECM metabolism. Based on these observations, I suggest that during wound healing and tissue remodeling the morphology of fibroblasts undergo a remarkable changes which in turn modulate their activity and ultimately control the quantity and quality of wound healing.

Figure II-1:

Immunocytochemical staining of anti-CC (a, b, e) and anti-LC (c, d, f) reactive TGF-ß1 in dermal fibroblasts. Cells were seeded for 4 h (a, c) or 24 h (b, d) on glass coverslips, fixed and processed as described in the text. Cells at the periphery of 24 h cultures (e, f) were stained positive.

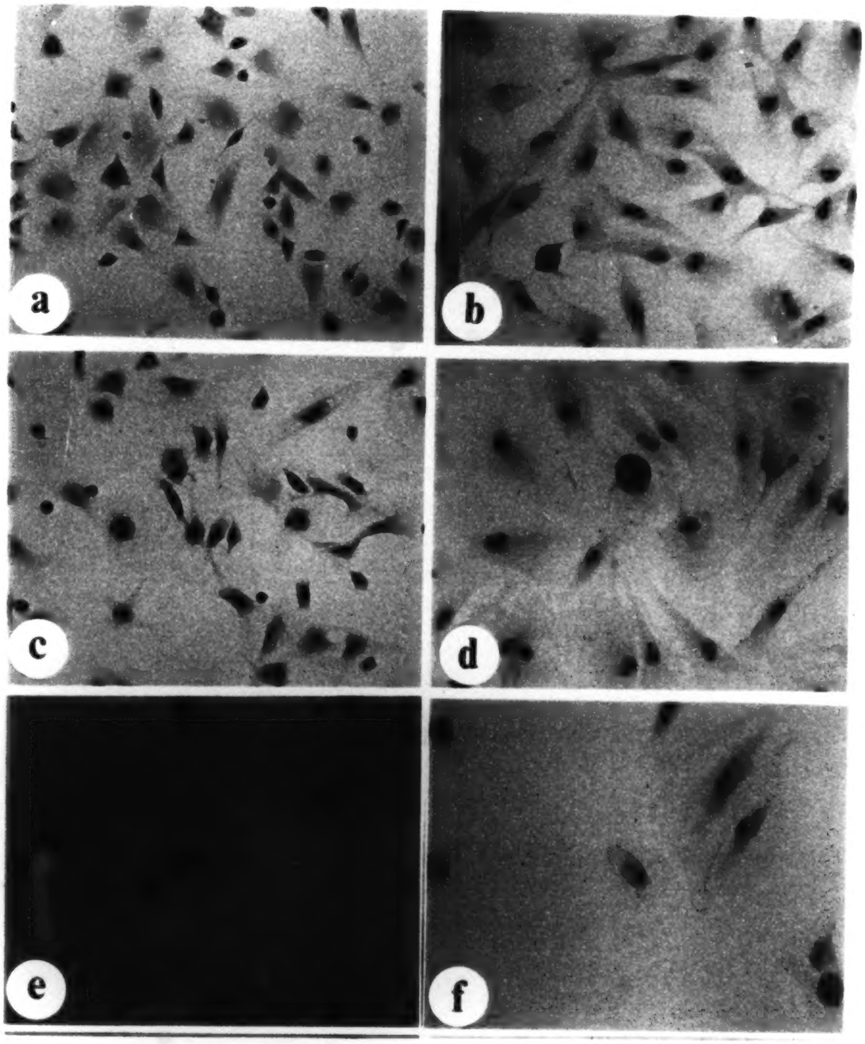


Figure II-2:

Rhodamine-labeled phalloidin staining of actin microfilaments in dermal fibroblasts grown on glass coverslips. Cells harvested by trypsin (a) or EGTA (b) and replated for 4 h. Confluent cells treated with Chs C (c). Cells seeded for 24 h (d).

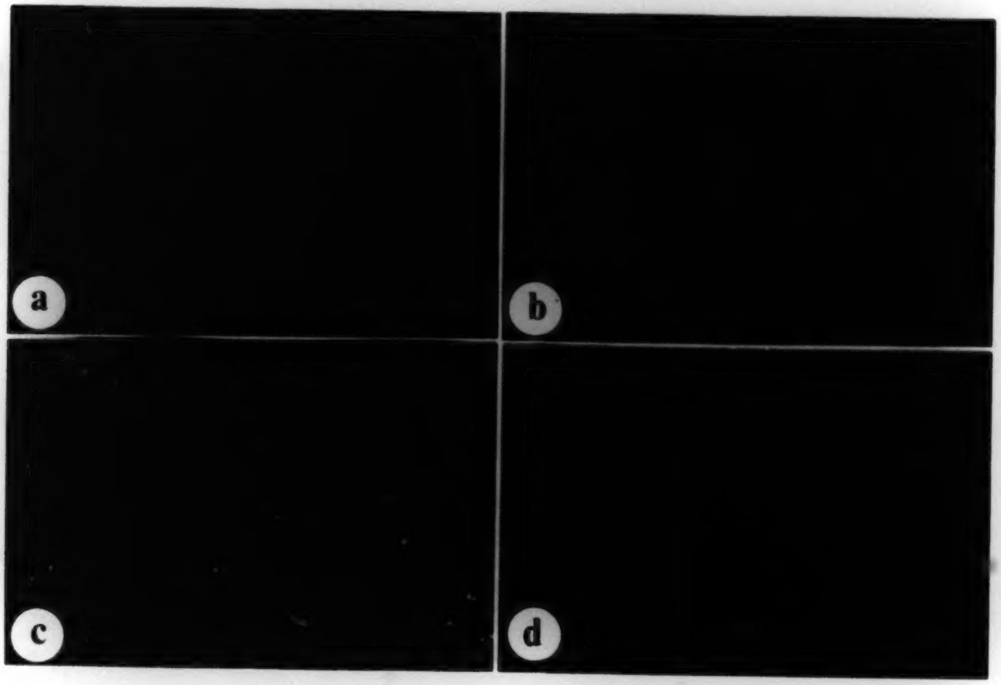


Figure II-3:

Expression of TGF-ß1 (2.5 Kb) and fibronectin (7.8 Kb) mRNAs in confluent (H, N) and 4 h-seeded (H', N') cells. A) Northern blot analysis of total RNA from HSc (H) and normal (N) fibroblasts. B) Fluorescence photograph of the corresponding ethidium bromide-stained gel. C) Quantitative analysis of the autoradiograms. Each bar represents the mean ± SEM of 4 cell strains. HSc (solid, solid hatched bars), normal (open, open hatched bars), confluent (solid and open bars) and trypsinized (hatched bars) cells. $^*p < 0.05$.

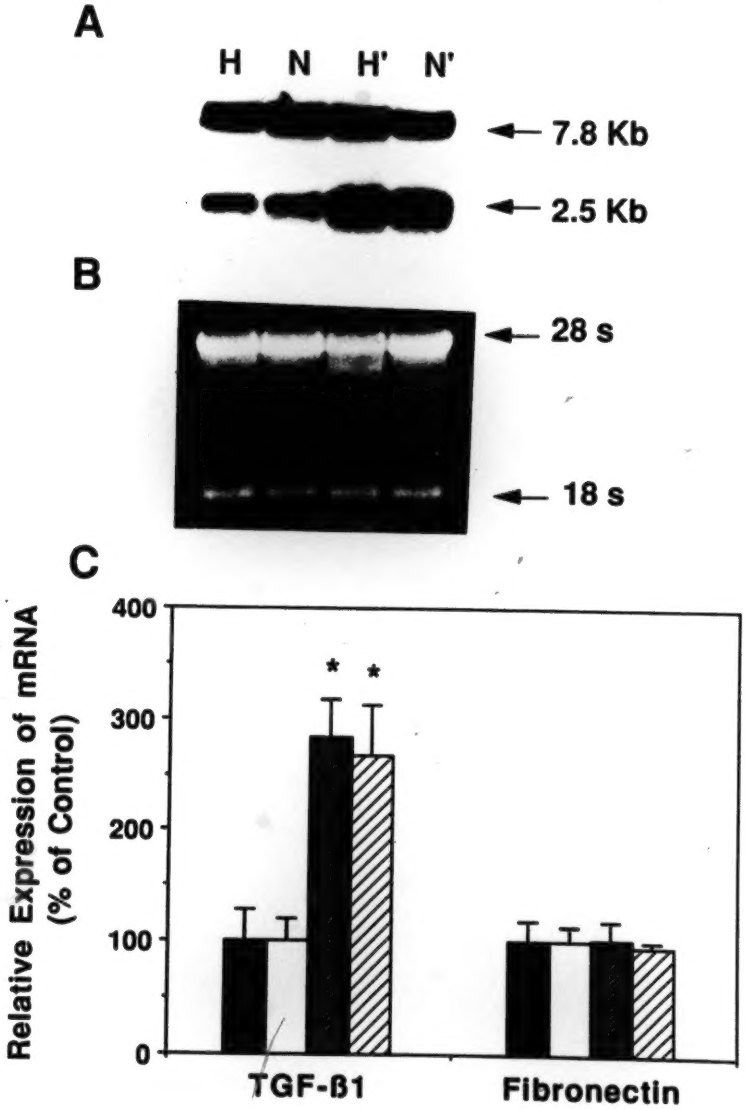


Figure II-4:

Expression of fibronectin (7.8 Kb) and TGF-ß1 (2.5 Kb) mRNAs in HSc (H) and normal (N) fibroblasts. A) Northern analysis of RNA from confluent (H, N), EGTA-treated (HE, NE) and Chs-treated (HC, NC) cells. B) Fluorescence photograph of the ethidium bromide-stained gel. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 cell strains. HSc (solid, solid hatched, solid dotted bars), normal (open, open hatched, open dotted bars), EGTA-treated (hatched bars) or Chs-treated (dotted bars), confluent (solid, open bars) cells. * $p < 0.05$.

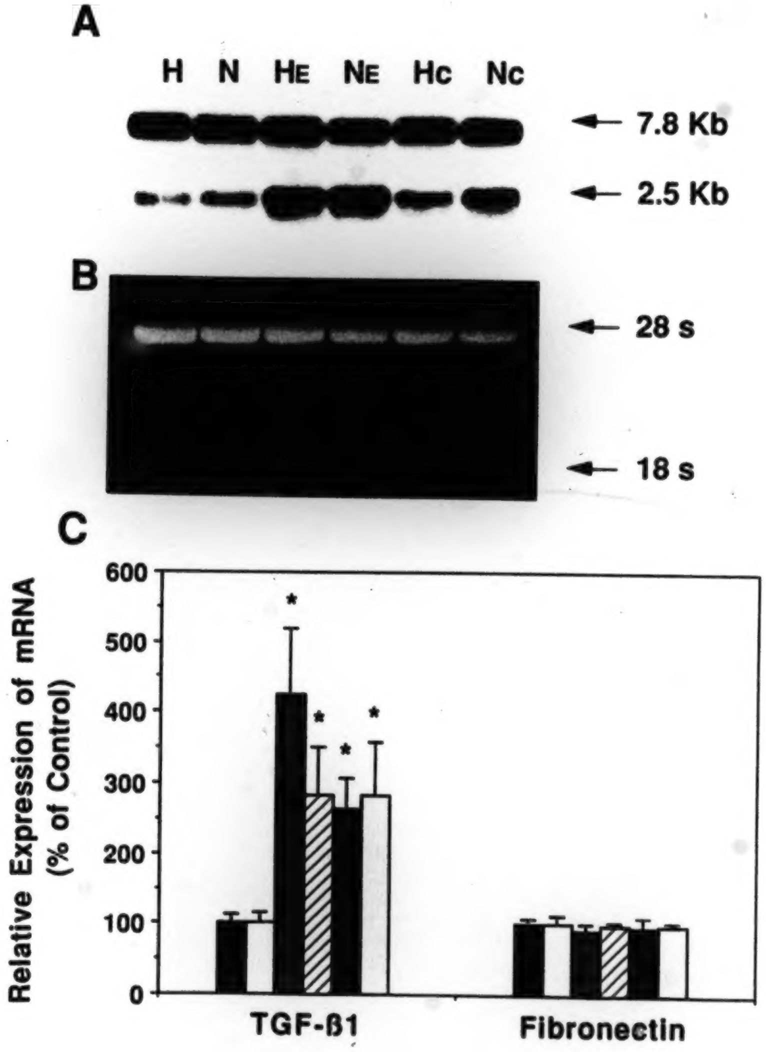


Figure II-5:

Time course of expression of TGF-ß1 mRNA in fibroblasts following disruption of the cytoskeleton. A) Northern analysis of total RNA from trypsinized cells plated for 4, 8, 12, 24, and 96 h (confluent culture). B) the corresponding fluorescence photograph of ethidium bromide- stained gel. C) Quantitative analysis of autoradiograms. Each bar shows the mean ± SEM of 3 tested cell strains. * $p < 0.05$.

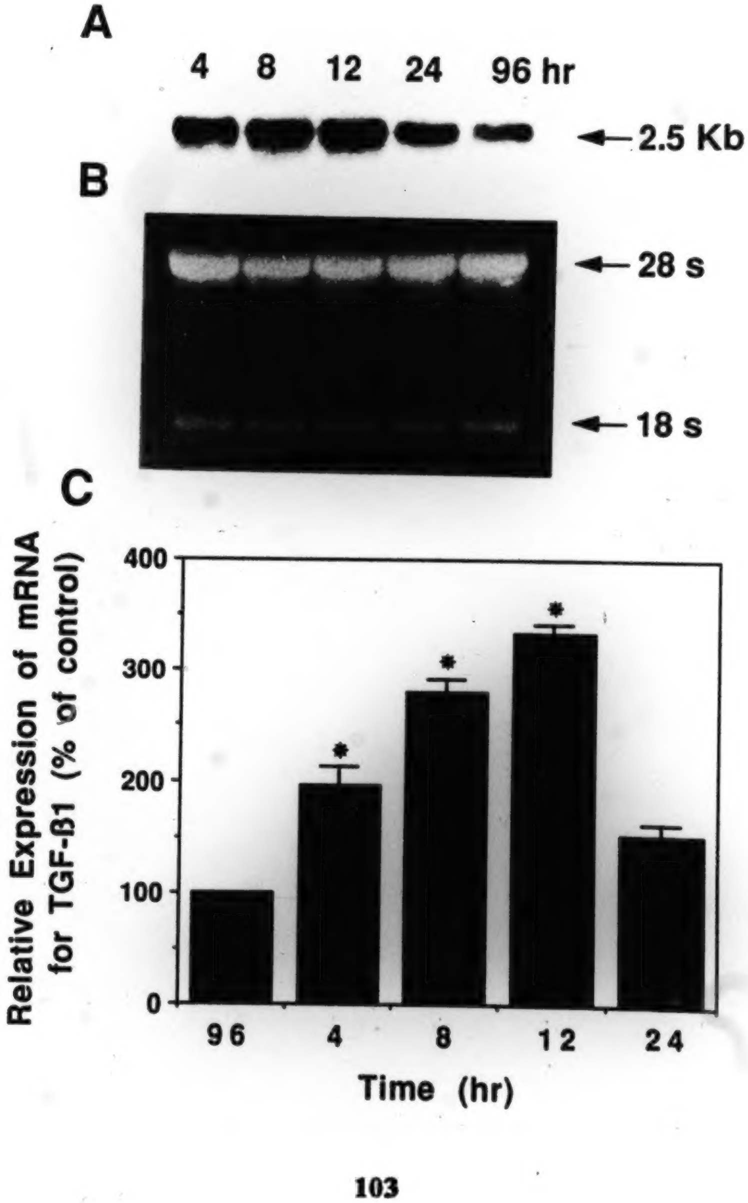


Figure II- 6:

Expression of mRNA for type I procollagen (α1, 5.8 and 4.8 Kb, faint and intense bands respectively), collagenase (2.1 Kb) and TIMP-I (0.7 Kb) in confluent (H, N) and trypsinized cells (H', N'). A) Northern blot analysis of RNA from HSc (H) and normal (N). B) the fluorescence photograph of the corresponding ethidium bromide-stained gel.
C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 tested cell strains. HSc (solid, solid hatched bars), normal (open, open hatched bars) confluent (solid, open) and trypsinized (hatched) cells. * $p < 0.05$.

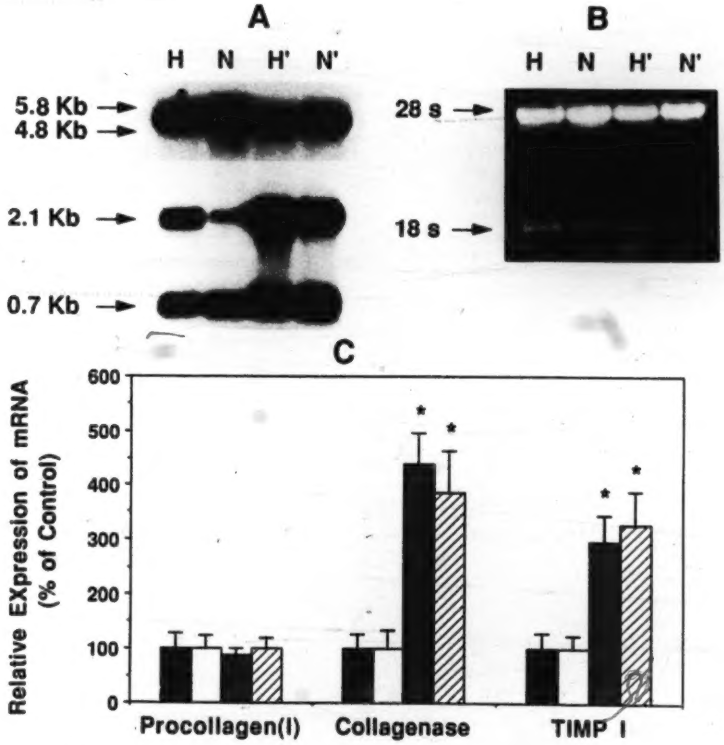
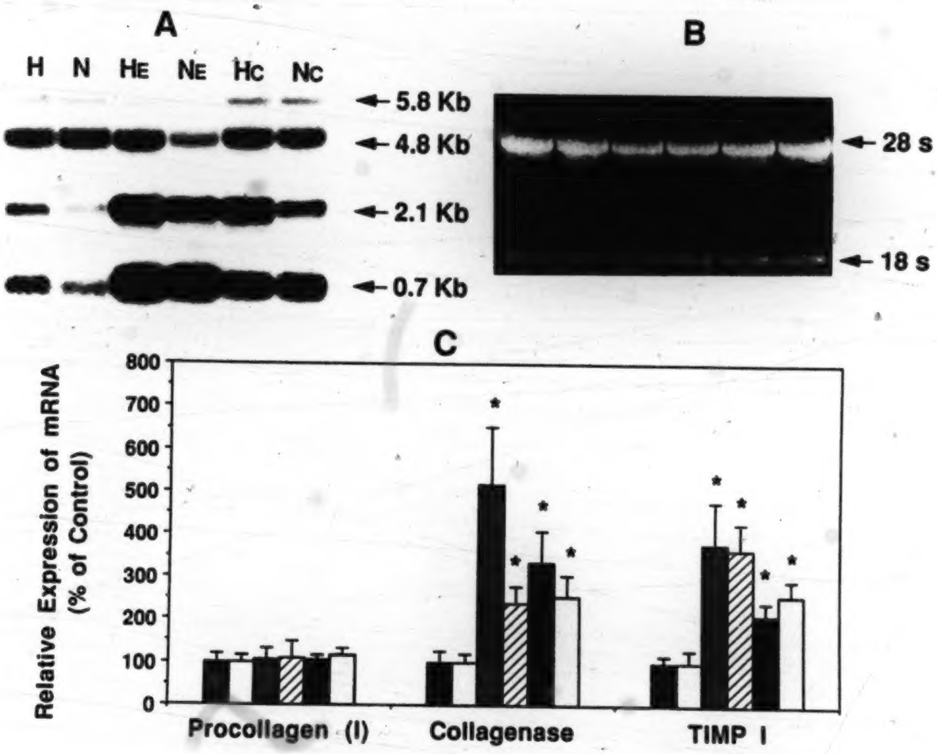


Figure II-7:

Expression of mRNA for procollagen I (α1, 5.8 and 4.8 Kb), collagenase (2.1 Kb), and TIMP-I (0.7 Kb) in confluent (H, N), EGTA-treated (HE, NE) and Chs-treated (HC, NC) cells. A) Northern analysis of total RNA from HSc (H) and normal (N) fibroblasts. B) Fluorescence photograph of the ethidium bromide-stained gel. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 cell strains. HSc (solid, solid hatched, solid dotted bars), normal (open, open hatched, open dotted bars), confluent culture (solid, open bars), EGTA-treated (hatched bars) and Chs-treated (dotted bars). * $p<0.05$.



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III

CYTOSKELETON REGULATES EXPRESSION OF GENES FOR TRANSFORMING GROWTH FACTOR-ß AND EXTRACELLULAR MATRIX PROTEINS IN DERMAL FIBROBLASTS **

INTRODUCTION

Fibroblasts are the major cell population responsible for the deposition, maintenance and remodeling of the extracellular matrix (ECM). These cells normally migrate to the site of the wound shortly after tissue injury, divide and begin to repair the damaged tissue (Clark, 1996). In recent years it has become apparent that upon activation these cells can also be a rich source of cytokines and growth factors, including transforming growth factor-beta1 (TGF-ß1). TGF-ß1 is a member of a large TGF-ß gene family which is conserved from *Drosophila* to humans (Padgett et al., 1987). Although the initial characterization of TGF-ß was based on its ability to induce transformation in normal rat kidney fibroblasts (Roberts et al., 1981), it is now clear that one of the most striking effects of TGF-ß is its ability to stimulate ECM deposition

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and connective tissue formation. Moreover, TGF-ß1 exhibits a variety of biological effects that are consistent with its proposed role in wound healing and tissue remodeling, such as the stimulation of granulation tissue formation and increasing the tensile strength of healing wounds (Mustoe et al., 1991). TGF-ß1 stimulates the synthesis of major matrix components including collagens and fibronectin by increasing the rate of transcription and stabilizing the transcripts (Ignotz et al., 1986). This fibrogenic factor also alters the degradation of matrix proteins by regulation of proteinases and proteinase inhibitors that affect the turnover of the ECM proteins. It has been shown that TGF-ß1 stimulates production of plasminogen activator inhibitor (Laiho et al., 1986a) and tissue inhibitor of metalloproteinase (TIMP) and down-regulates the production of collagenase (Edwards et al., 1987) and plasminogen activator (Laiho et al., 1986b). These biological activities lead to accumulation and stabilization of extracellular matrix. Appropriate expression and function of ECM structural proteins, matrix metalloproteinases (MMPs), TIMPs, cytokines and growth factors are essential to normal tissue repair. TGF-ß1 is involved in the regulation of these components and, therefore, plays a major role in the outcome of the tissue repair process (Shah et al., 1995).

Despite intensive investigation into the biologic activities of TGF-ß1, the mechanisms by which expression of this growth factor is regulated are not yet fully understood. Several factors such as differentiation (Loveridge et al., 1993), oxygen tension (Falanga et al., 1991), members of the steroid hormone superfamily (Wakefield et al., 1990), immunosuppressive drugs such as cyclosporine A (Ahuja et al., 1995), retinoic acid receptors (Salbert et al., 1993) and ECM components (Streuli et al., 1993) have been shown to influence TGF-ß1 expression. It has also been shown that TGF-ß1 regulates its own expression in an autocrine manner (Obberghen-Schilling et al., 1988).

I have recently reported that re-organization of the cytoskeleton induces TGF-ß1, collagenase and TIMP-I mRNAs in dermal fibroblasts (Varedi et al., 1995; Chapter II). The cytoskeleton has been shown to be involved in the transduction of extracellular signals to the nucleus through both physical and chemical pathways (Bissell et al., 1982; Ben-Ze'ev, 1991) and thereby to control expression of specific genes. The interaction between the cytoskeleton and the ECM is mediated through the integrins and a set of protein complexes collectively called focal adhesion plaques (Burridge and Mangeat, 1984). Although the actual role of the cytoskeleton in signal transduction and gene expression is unclear, the association of different components of the signal transduction cascade, particularly focal adhesion kinase (P 125 $^{\text{FAK}}$), with the focal adhesion plaques (Zachary and Rozengurt, 1992), supports the idea that re-organization of cytoskeleton can play a crucial role in signal transduction and gene expression. From a physical standpoint, focal adhesion plaques link the cytoskeleton to the ECM. The ECM thus not only serves as a scaffold for cells but also controls cell morphology and regulates many cellular functions via the cytoskeleton. The ECM is a dynamic environment during tissue repair (Clark, 1996), influencing the morphology of the resident cells including fibroblasts. The ECM-mediated alterations in cell morphology could affect the insoluble nuclear matrix that regulates gene expression and ultimately cell function. The aim of this investigation was to examine the effect of re-organization of the cytoskeleton of dermal fibroblasts on transcription of the genes for ECM structural proteins (procollagen I and fibronectin), proteins involved in ECM turnover (collagenase and TIMP-I) and a regulatory growth factor (TGF-ß1). Since activation of the gene for TGF-ß1 is believed to be largely governed by the AP1 complex binding elements located in the promoters of this gene (Kim et al., 1989b), the expression of mRNAs for *c-fos* and *c-jun* was also evaluated. Disruption of cytoskeleton was induced by trypsin, EGTA or Chs C and the nuclear run-on assay was used to evaluate the rate of gene transcription. These results show that the status of the cytoskeleton influences the expression of genes for proteins involved in the homeostasis of ECM.

MATERIALS AND METHODS

cDNA Probes, Plasmids DNA and Reagents

The complementary DNA (cDNA) probe for TGF-ß1 was characterized by Ardinger et al. (1988) and donated by Dr. G.I. Bell, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, Illinois, USA. The type I procollagen (α1 and α2) cDNAs were characterized and provided by Drs. G. Tromp (1988), H. Kuivaniemi (1988), and L. Ala-Kokko (1989, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA). The cDNAs for fibronectin (Bernard et al., 1985), TIMP-I (Durfy et al., 1986), 18 S rRNA (Young et al., 1976; McCallum and Maden, 1985), collagenase (Witham et al., 1986) and *c-jun* (Ryder and Nathans, 1988) were purchased from American Type Culture Collection (ATCC, 12301, Parklawn Drive, Rockville, MD, 20852, USA). The *c-fos* probe was a 1 Kb Pst I fragment of the human *fos* genomic DNA (Curran et al., 1983) purchased from ATCC. The plasmids carrying the specific cDNA for the genes of interest were amplified and after purification and immobilization on nitrocellulose were used as probes in the nuclear run-on experiments. EGTA (ethyleneglycol-bis (ß-amino ethyl ether) tetra acetic acid), cytochalasin C (Chs), cycloheximide (Cxm) and actinomycin D (Act. D) were purchased from Sigma (St. Louis, MO). RNase-free DNase I, dithiothreitol (DTT) and tRNA were purchased from Boehringer Mannheim (Germany). Proteinase K, ATP, CTP and GTP were purchased from Gibco BRL (Gaithersburg, MD). The [α–^{32}P] UTP and [α–^{32}P] dCTP were purchased from DuPont Inc. (Mississauga, Ontario). Nitrocellulose membrane (NitroPure) was purchased from MSI (Westboro, MA).

Cell Culture and Treatments

Cultures of fibroblasts from dermis were established according to Nakano and Scott (1986) as described in Chapter II. In this study, I used fibroblast cultures at passages 6 and 7. Disruption of the cytoskeleton was induced by either trypsin, EGTA, or cytochalasin C (Chs) as previously described (Varedi et al., 1995; Chapter II). Briefly, fully confluent cells were harvested from culture flasks using either 3 ml of 0.1% (w/v) trypsin and 0.02% (w/v) EDTA in PBS (pH 7.4) for 3 min or 8 ml of 0.05% (w/v) EGTA in Hanks' Balance Salt Solution (HBSS) for 30 - 45 min. Cells were washed twice in DMEM + 10% (v/v) FBS, re-suspended in the same medium, split 1:3 and replated in fresh culture flasks. Cultures were then incubated at 37° C in a humidified atmosphere of 5% CO_2 in air for the indicated time. At the end of the incubation period, cells were washed with ice-cold PBS and used for extraction of total RNA or isolation of the nuclei. Cells were resuspended in serum-free medium when the expression of protooncogene mRNAs was to be examined. When Chs was used, fully confluent cells were treated with Chs (1 - 2 µg/ml) and incubated for the indicated times. Cells were then washed with cold PBS and used for extraction of the total RNA or isolation of the nuclei. Cytochalasin has been shown to inhibit protein synthesis at concentrations equal to or greater than 4 µg/ml (Ornelles et al., 1986). To avoid this undesired side effect, I have used a lower dose of Chs which is nevertheless adequate to disrupt the cytoskeleton. The viability of Chs-treated cells was greater than 90%, as judged by exclusion of trypan blue dye and by recovery of cells following replacement of medium lacking Chs. The organization of the cytoskeleton was visualized by rhodamine-labeled phalloidin staining, as previously described (Varedi et al., 1995; Chapter II). The Cxm and Act. D were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml and 10 mg/ml respectively and then used at a final concentrations of 20 µg/ml and 5 µg/ml respectively. Both reagents were prepared fresh for each experiment. When Cxm and/or Act. D were used, the control cells received an equal volume of the vehicle DMSO.

RNA Extraction and Northern Analysis

Fibroblast cell layers were lysed and pooled in 6 ml of 4 M guanidinium isothiocyanate (GITC) and total RNA from each individual cell lysate was isolated by the GITC/CsCl procedure of Chirgwin et al. (1979) as described in Chapter II. Extracted total RNA from each individual fibroblast culture was then separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto a nitrocellulose membrane. To ensure that the gel lanes were loaded with similar amounts of total RNA, the quantity of 18 S and 28 S ribosomal RNA were compared visually by ethidium bromide fluorescence and when the loading of total RNA was more than 10 µg/lane, the blot was probed with a ^{32}P-labeled cDNA specific for 18 S ribosomal RNA. The blots were baked for 2 h at 80° C under a vacuum and then pre-hybridized for 4 - 6 h at 42° C in a prehybridization solution containing 50% formamide, 20 mM NaH_2PO_4 (pH 7.0), 4x SSC, 2 mM EDTA, 4x Denhardt's solution (1x = 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 1% (w/v) SDS, and 100 mg/ml sonicated denatured salmon sperm DNA. Hybridization was then performed at 42° C in the same solution for 16 - 20 h using either human TGF-ß1, *c-jun*, *c-fos*, 18 S rRNA or collagenase cDNA probes. The cDNA probes were labeled with ^{32}P-alphaα–dCTP by nick-translation. The membranes were washed at room temperature with 2x

SSC and 0.1% (w/v) SDS for 1 h and then washed for 30 min at 65° C in 0.2x SSC and 0.1% (w/v) SDS. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose membranes at -70° C in the presence of an intensifying screen. Autoradiographs and the corresponding ethidium bromide fluorescence photographs were scanned using a Color One Scanner (Macintosh, USA) and quantitated using NIH Image software (Version 1.75). Loading of total RNA was also normalized using the fluorescence intensity of ethidium bromide.

Nuclear run-on Assay

The nuclei were prepared using a modified procedure described by Bentley and Groudine (1986). Briefly, fibroblast cell layers were harvested by scraping in ice-cold PBS, centrifuged, washed with a re-suspension buffer (10 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.4) and recentrifuged. The cell pellet was resuspended in the same buffer containing 0.5% (v/v) NP40 (Nonidet), shaken for 5 min and the nuclei were isolated by centrifugation at 2500 rpm for 10 min at 4° C. Isolated nuclei were then resuspended in nuclear freezing buffer (50 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, and 40% glycerol), counted under a light microscope using a hemocytometer and stored in liquid nitrogen. *In vitro* nuclear run-on transcription was accomplished according to a modified protocol of Greenber and Ziff (Greenber and Ziff, 1984). Each reaction typically contained 5 x 10^7 nuclei (210 µl), 250 µCi [α–^{32}P] UTP, 0.33 mM ATP, CTP and GTP, in a final volume of 295 µl. The reaction mixture was incubated for 30 min at 30° C with intermittent shaking. To isolate the radiolabelled RNA, the reaction mixture was treated with RNase-free DNase I (300 unit), for 5 min at 30° C followed by treatment with proteinase K (100 µg) for 45 min at 45° C. The RNA was then extracted with phenol/chloroform-/isoamyl alcohol, precipitated by adding 2.2 volumes of cold ethanol and 0.1 volume of sodium acetate (3 M, pH 5.2) and stored overnight at -20° C. The precipitated RNA was resuspended in TE buffer (1 M Tris, and 0.5 M EDTA, pH 8.0) and the radioactivity measured. The plasmid vectors, carrying the cDNA for the indicated genes (20-25 µg) were immobilized onto nitrocellulose filters using a Bio-Rad slot blot apparatus. The blots were baked for 2 h at 80° C under vacuum and then prehybridized for 8 h at 55° C in 3 ml of hybridization solution (10 mM MOPS pH 7.4, 1% w/v SDS, 10 mM EDTA, 300 mM NaCl, 1x Denhardt's solution, 0.25% w/v non-fat dry milk and 250 µg/ml of tRNA). Hybridization was performed for 24 h at 55° C in the same solution by the addition of the corresponding newly synthesized radiolabelled RNAs (2 x 10^6 cpm/ml). The blots were then washed initially in 2x SSC and 0.1% (w/v) SDS for 30 min at room temperature and then in 0.2x SSC and 0.05% (w/v) SDS for 15 min at

60° C. Autoradiography andquantitative analysis of the autoradiographs were performed as described for Northern analysis.

Statistical Analysis

The data were analyzed using a paired two-tailed t - test to determine if differences were significant. $p < 0.05$ was considered significant.

RESULTS

Disruption of cytoskeleton by trypsin, EGTA or Chs increases the rate of transcription of TGF-ß1 and collagenase genes As shown in Figure III-1, confluent cells harvested by trypsin or EGTA and replated for 3 - 4 h, which allowed re-assembly of the cytoskeleton, confirmed by rhodamine-labeled phalloidin staining of the filamentous actin (data not shown), showed 2.0 fold and 2.7 fold increases in transcription of the TGF-ß1 gene respectively. The rate of transcription of the collagenase gene was increased 5.3 fold by trypsin and 6.2 fold by EGTA. I observed a similar effect when the cytoskeleton was disrupted by Chs. Fully confluent cells treated with Chs for 4 h, which caused disassembly of the cytoskeleton microfilaments as confirmed by rhodamine-labeled phalloidin staining, showed 1.6 fold and 3.7 fold increases in the rate of transcription of the TGF-ß1 gene and collagenase gene respectively.

The status of cytoskeleton differentially regulates ECM structural protein, and TIMP-I gene transcription As shown in Figure III-1, The rate of transcription of the TIMP-I gene was increased 4.6 fold by trypsin and 3.8 fold by EGTA. A slight increase, 1.1 fold, in the rate of transcription of this gene was observed after treatment with Chs. Interestingly, Chs increased the rate of transcription of procollagen I (α1 and α2) and fibronectin genes by 1.4 fold 1.5, fold and 1.9 fold respectively, while trypsin or EGTA had almost no effect on the rate of transcription of these genes and only slight effect on the fibronectin gene.

Trypsinization induces AP1 complex component (c-jun and c-fos) mRNAs The promoter region in the TGF-ß1 gene, as in many other genes, contains several putative AP1 binding elements which have been shown to be largely involved in its regulation (Kim et al., 1990). The collagenase and TIMP-I gene promoters also possess AP1 binding elements which are involved in the regulation of the transcription of these genes (Edwards et al., 1992). Since an increase in the rate of transcription of TGF-ß1, collagenase and TIMP-I genes has been observed following disruption of cytoskeleton by trypsin or EGTA, I examined the expression of mRNAs for the AP1 complex components, *c-fos* and *c-jun*, following disruption of the cytoskeleton by trypsin. Based on the rhodamine-labeled phalloidin staining, dermal fibroblasts harvested by trypsin or EGTA and replated for a short period of time exhibit a similar cell morphology and are identical, at least physically, in the organization of cytoskeleton microfilaments (data not shown). I chose trypsin over EGTA since a large number of cells was required for these experiments and I could obtain more cells from confluent cultures using trypsin. Confluent cells were trypsinized and replated in serum-free medium for the indicated times. As shown in Figure III-2, the expression of TGF-ß1 mRNA (2.5 Kb) was increased 1.6 fold as early as 1 h and peaked (2.7 fold increase) at 8 h and decreased 24 h after trypsinization. The *c-fos* mRNA (2.7 Kb) was observed 30 min following disruption of the cytoskeleton but it could not be detected at later times. The expression of both, 3.2 Kb and 2.7 Kb, *c-jun* transcripts (Pertovaara et al., 1989) was increased by 2.4 fold 4 h after treatment and persisted for at least 24 h.

The turnover of TGF-ß1 mRNA is affected by the status of cytoskeleton

As shown in Figure III-3, A, inhibition of new RNA synthesis by Act. D blocked the induction of TGF-ß1 mRNA by Chs. Confluent cells were treated with Chs and Act. D for the indicated time. Northern blot analysis showed that Act. D suppressed the TGF-ß1 mRNA after 0.5, 1, 3, and 6 h by 58%, 66%, 60% and 58% respectively. Actinomycin had less effect on the level of TGF-ß1 mRNA in trypsinized cells. Confluent cells were trypsinized and incubated for 4 h before the addition of Act. D. Northern blot analysis showed that Act. D suppressed the TGF-ß1 mRNA after 0.5, 1, 3, and 6 h by 44%, 41%, 34% and 41% respectively (Figure III-3, B). Confluent cells treated with Act. D for the indicated times served as controls (Figure III-3, A, B). After 6 h of Act. D treatment the level of TGF-ß1 mRNA in confluent control cells dropped by 35% and 41% (panels A and B respectively) showing that the half life of TGF-ß1 mRNA is apparently greater than 6 h in these cells.

TGF-ß1 mRNA but not collagenase mRNA is super - induced by inhibition of protein synthesis As shown in Figure III-4, panel A and C, Chs significantly increased the 2.5 Kb mRNA for TGF-ß1 by 47.8 ± 12% ($n = 5$, $p<0.05$) after 6 h as compared to confluent cells (lane Cont). When fibroblasts were treated with Chs and Cxm simultaneously (lane Chs+Cxm), a super-induction of TGF-ß1 mRNA by 88 ± 23% ($n = 4$, $P<0.05$) was observed. Cycloheximide alone (lane Cxm) had no significant effect on TGF-ß1 mRNA. The super-induction of TGF-ß1 mRNA by Cxm seems to be selective since the Chs-induced collagenase mRNA (2.1 Kb, Panel A, lane Chs) was not super-induced by Cxm but rather totally blocked, indicating that an intact protein synthesis machinery is essential. Interestingly, prior exposure of cells to Act. D for 1.5 h (Panel A, lane Act D+Chs+Cxm) completely blocked the Cxm-induced super-induction of TGF-ß1 mRNA (2.5 Kb, panel A), suggesting that transcriptional activation is involved in this induction. Actinomycin totally blocked the induction of collagenase and TGF-ß1 mRNAs by Chs (Panel A, lane Chs+Act D), indicating that these inductions are mediated through transcriptional activation, consistent with the results of the nuclear run-on assay. I could not examine the effect of inhibition of protein synthesis by Cxm in trypsinized cells because such cells do not adhere effectively in the presence of Cxm.

DISCUSSION

This study provides evidence that the status of the cytoskeleton can regulate expression of specific genes at the level of both transcription and stability of mRNA. I have previously reported a significant increase in the level of TGF-ß1, collagenase and TIMP-I mRNAs following disruption of cytoskeleton (Varedi et al., 1995; Chapter II). The nuclear run-on results indicate that this induction is mediated, at least in part, at the level of gene transcription. The rate of transcription of TGF-ß1 and collagenase genes was increased by each of the agents used to disrupt the cytoskeleton. However, the level of induction was different for each agent and EGTA was a more effective inducer. Although I have not examined the role of Ca^{++} and protein kinase C in the regulation of TGF-ß1 and collagenase genes, this effect could possibly be due to alteration in the intracellular Ca^{++} which is a potent mediator of this signal transduction pathway. The potent protein kinase C activator, 12-O-tetradecanoylphorbol acetate, activates both collagenase and TGF-ß1 genes (Kim et al., 1989a; Angel et al., 1987). Trypsin and EGTA had similar effects on the genes examined in this investigation but Chs had different effects on TIMP-I, type I collagen ($\alpha1$, $\alpha2$) and fibronectin genes. Trypsin, EGTA and Chs interfere with cell-matrix interactions and disrupt the cytoskeleton through different mechanisms. Trypsin nonspecifically cleaves the ectodomains of adhesion molecules (integrins), while EGTA specifically chelates Ca^{++} thereby inhibiting Ca^{++}- dependent interaction of cell and matrix. However, cytoskeleton starts to recover in both trypsinized and EGTA-treated cells shortly after seeding and a few hours later the organization of microfilaments appears identical (Varedi et al., 1995; Chapter II). Chs specifically binds to the fast - growing end (plus end) of filamentous actin and shifts the kinetics of assembly towards dissociation (Cooper, 1987). Cells treated with Chs showed no sign of assembled microfilaments (stress fibers), while shortly after seeding, trypsinized or EGTA- treated cells re-organized their cytoskeleton. Although trypsin, EGTA or Chs may have other effects on cell function which ultimately affect gene expression, I suggest that the differential effects of these agents are due, at least in part, to differences in the status of the cytoskeleton and cell morphology. I have previously shown that trypsin, EGTA or Chs had no significant effects on the level of procollagen I (α1) or fibronectin mRNAs (Varedi et al., 1995; Chapter II). Consistent with Northern analysis, the nuclear run-on results in the current study indicated that trypsin or EGTA had minor effects on the transcription of these genes. However, Chs increased the transcription of these genes, particularly that of fibronectin. This discrepancy could be due to destabilization of the transcripts as was apparently the case for TGF-ß1 (see below).

The TGF-ß1 promoter contains several AP1 binding elements and autoinduction of the TGF-ß1 gene and activation by phorbol ester have been shown to be mediated through these elements (Kim et al., 1989a, 1989b, 1990). The AP1 complex is either a heterodimer of *c-fos* and *c-jun* or a homodimer of *c-jun* protooncoproteins (Halazonetis et al., 1988). The degradation of *c-fos* mRNA occurs extremely rapidly (half - life <10 min) but the *c-fos* protein and its complex with *jun* protein are believed to be relatively stable (Muller et al., 1988). The early induction of TGF-ß1 in trypsinized fibroblasts, however, may be independent of the AP1 complex, since TGF-ß1 mRNA was induced before *c-jun* mRNA. The later peak in TGF-ß1 mRNA may be partly due to autoinduction mediated through the AP1 complex, particularly *jun*. (Kim et al., 1989a). Information on *c-fos* and *c-jun* protein levels would be of interest in this regard. Although I do not have enough evidence to prove that these elements are involved in this induction, the time course of expression of *c-fos* and *c-jun* mRNA is compatible with this suggestion.

The status of the cytoskeleton seems to control the stability of the TGF-ß1 transcript. Although inhibition of transcription by Act. D suppressed the expression of TGF-ß1 mRNA in both trypsinized cells and Chs-treated cells, the effect was more pronounced in the latter. Moreover, Cxm which is known to be an mRNA stabilizer (Sorrentino et al., 1985), super-induced TGF-ß1 mRNA in Chs-treated cells, indicating that the half-life of TGF-ß1 mRNA is reduced in these cells. This discrepancy can also be attributed to the status of the cytoskeleton which is substantially different in trypsinized cells replated for a short time from that in cells treated with Chs. Pachter (1992) has provided evidence that the majority of cellular mRNA is associated with the cytoskeleton. This association appears to control the stability as well as the topological arrangement and spatial segregation of different mRNAs which can ultimately influence the efficiency of their translation.

Inhibition of protein synthesis by Cxm is a widely used approach to examine the involvement of newly-synthesized regulatory molecules in the activation of specific genes. However, it has been shown that this antibiotic not only blocks the elongation phase of translation but also increases gene expression, steady-state levels of mRNA and the levels of certain proteins (Ringold et al., 1984; Sorrentino et al., 1985). Cycloheximide-mediated super-induction of several mRNAs, particularly cytokines and growth factors, has been reported (Takane et al., 1993). A super-induction of TGF-ß1 mRNA has been observed during autoinduction (Obberghen-Schilling et al., 1988). I found similar results when the alterations in cell morphology were induced by Chs in the presence of Cxm. I have also shown that the Cxm-induced super-induction of mRNA is selective. Although the exact mechanism(s) of this effect is (are) not yet clear, it has been attributed, in part, to a decrease in RNase synthesis which would lead to less degradation of the mRNAs (Ringold et al., 1984). Sorrentino et al. (1985) have shown that inhibition of protein synthesis by Cxm leads to the production of molecules that can positively regulate gene expression. These results support the idea that in addition to stabilizing mRNAs, Cxm may affect the transcriptional activity of specific genes. One may also speculate that Cxm inhibits the synthesis of short-lived gene repressor proteins and activates gene transcription by removing the inhibitory effects of these regulators.

CONCLUSION

The data presented here are of interest in relation to several physiological processes, such as embryogenesis and wound healing, in which the expression of ECM structural proteins, proteins involved in the turnover of ECM and growth factors act in concert. Evidence from both *in vivo* and *in vitro* investigations supports the idea that loss of the coordination of the expression of these ECM proteins during tissue repair either impairs healing or leads to fibrosis. In conclusion, the data suggest that the cytoarchitecture of dermal fibroblasts regulates expression of genes for protein involved in the structure (type I collagen and fibronectin), turnover (collagenase and TIMP-I) and regulation (TGF-ß1) of ECM. Since the cytoarchitecture undergoes remarkable changes during cell division, migration, and differentiation, these results may have implications for the regulation of ECM during such processes as embryogenesis, carcinogenesis and wound healing.

Figure III-1:

Disruption of the cytoskeleton activates transcription of specific genes in dermal fibroblasts. Representative autoradiograph of a nuclear run-on assay performed following disruption of cytoskeleton by trypsin, EGTA or Chs C (Chs). Confluent cells treated with fresh media for 4 h were used as a control (Cont). The isolated nuclei were subjected to nuclear run-on *in vitro* transcription. The newly synthesized radiolabelled RNA was isolated and hybridized to plasmid DNAs on a nitrocellulose membrane for the indicated genes. The pGEM4 plasmid DNA without insert (vector) was used as a negative control for hybridization.

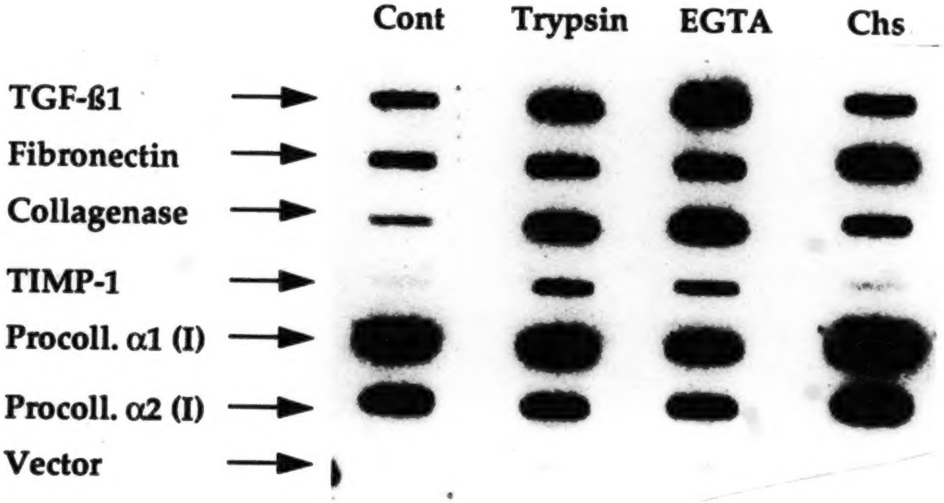


Figure III-2:

Disruption of cytoskeleton induces expression of TGF-ß1, *c-fos* and *c-jun* mRNAs. Representative Northern blot analysis of TGF-ß1 (2.5 Kb), *c-fos* (2.7 Kb) and *c-jun* (3.2 Kb and 2.7 Kb) mRNAs from dermal fibroblasts following disruption of cytoskeleton. Confluent cells were trypsinized and replated in serum-free medium for the indicated times. Confluent cells treated with serum-free medium for 24 h were served as a control (Cont). Total RNA was extracted from the cells at each time and subjected to Northern analysis (35 µg/lane). Following hybridization with TGF-ß1, and *c-jun* cDNAs, the blot was stripped and rehybridized with specific cDNAs for *c-fos* and 18 S ribosomal RNA (as a control for loading).

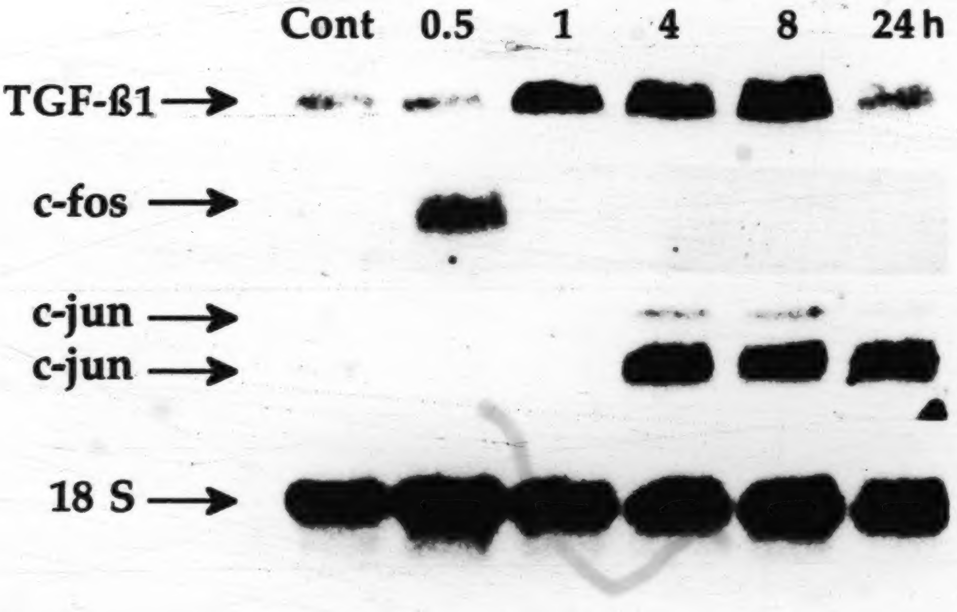


Figure III-3:

The TGF-ß1 mRNA is stabilized in trypsinized cells relative to Chs-treated cells. A) Confluent cells were treated with Chs (circular symbols) or fresh medium (triangular symbols) and incubated in the presence of Act. D for the indicated times. B) Confluent cells were subcultured by trypsinization (circular symbols) or treated with fresh media (triangular symbols) and incubated for 4 h. Act. D was then added and incubation proceeded for 0, 0.5, 1, 3 or 6 h. Total RNA was subjected to Northern analysis. The graphs show quantitative analysis of the autoradiograms. The mRNA expression was plotted relative to the levels of the mRNA at 0 time. The curves were fitted by hand.

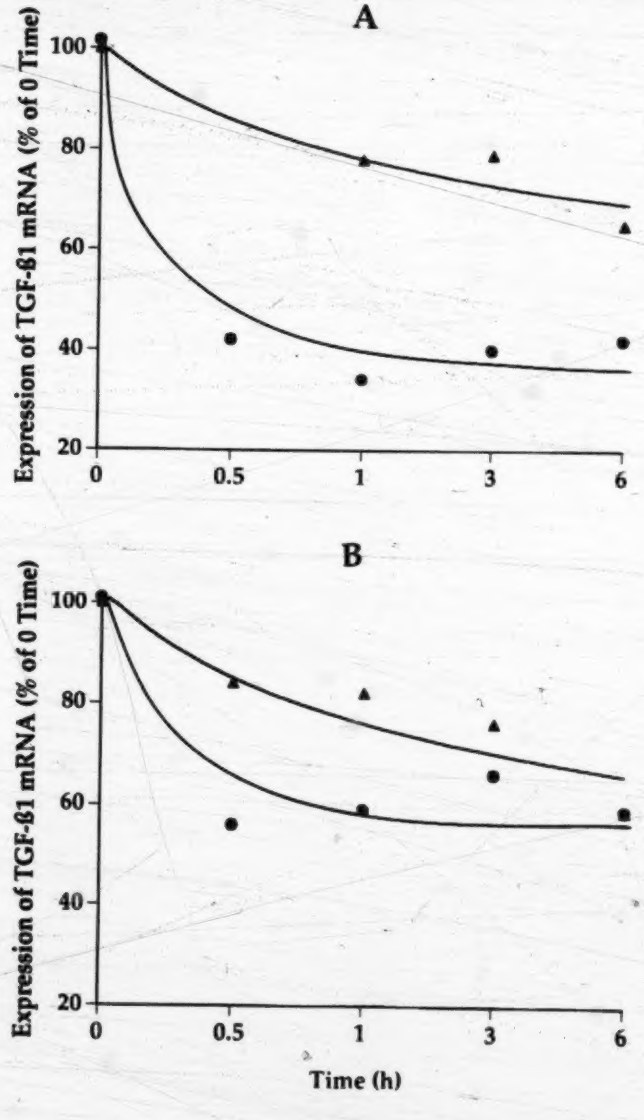
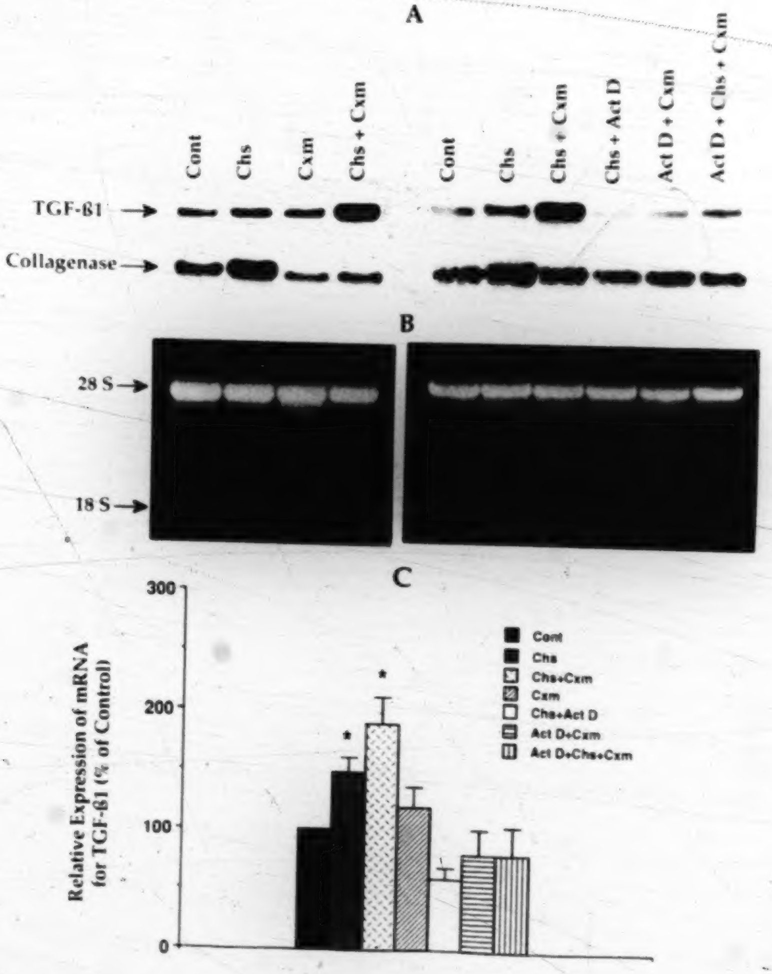


Figure III-4:

Superinduction of TGF-ß1 mRNA (2.5 Kb) but not collagenase mRNA (2.1 Kb) by Cxm in dermal fibroblasts. A) Northern analysis of total from confluent cells (Cont.), cells treated with Chs (Chs), Cxm (Cxm), Chs and Cxm (Chs+Cxm), Chs and Act. D (Chs+Act D), Act. D and Cxm (Act D+Cxm) for 4 h or preexposed to Act. D for 1.5 h and then incubated in the presence of Chs and Cxm for 4 h (Act D+Chs+Cxm). The first four lanes show the results of one experiment and the next six lanes the results of a second experiment. Both experiments included control and Chs-treated cells. B) Fluorescence photographs of the ethidium bromide stained gels. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 2-5 tested cell strains. * Statistically significant, $P<0.05$



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IV

RE-ORGANIZATION OF COLLAGEN MATRIX BY DERMAL FIBROBLASTS MODULATES MORPHOLOGY, GENE EXPRESSION, GROWTH AND APOPTOSIS *¥

INTRODUCTION

During many physiological processes the extracellular matrix (ECM) undergoes a series of changes in physicochemical properties that may subsequently affect morphology, behavior, growth and survival of the resident cells. For instance, shortly after cutaneous injury, fibroblasts migrate from the adjacent normal dermis to a plasma fibronectin/fibrin-rich provisional matrix or blood clot, where they begin to divide and replace the damaged matrix with another transient matrix known as granulation tissue (Kurkinen et al., 1980). Unlike the normal dermis, granulation tissue is hypercellular and hypervascular and contains high levels of locally-made fibronectin and an elevated level of type III collagen (Clark, 1996). Eventually, the granulation tissue remodels and the hypercellularity diminishes by cell death

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resulting in a mature scar (Desmouliere and Gabbiani, 1996). The repertoire of cytokines and growth factors, including TGF-ß, in granulation tissue is also different from that in normal dermis (Levine et al., 1993). Moreover, it has been shown that fibroblasts from wounds of different stages of healing or fibrotic tissue vary in morphology, cytoskeletal proteins, gene expression, and response to growth factors (Gabbiani et al., 1972 ; Skalli et al., 1989; Finesmith et al., 1990; Germain et al., 1994; McCaffrey et al., 1995). These reports support the idea that the physicochemical properties of ECM regulate fibroblast activity. Moreover, ECM can indirectly influence cell activity by acting as a 'reservoir' or 'sink' for growth factors that regulate many cellular functions.

Although two-dimensional monolayer culture of fibroblasts is commonly used, it has limited resemblance to the *in vivo* situation where fibroblasts are embedded in and interact with a three-dimensional and dynamic matrix consisting mainly of type I collagen. An *in vitro* model of fibroblast culture in a collagen matrix (CM) was established by Bell et al. in 1979. Since in this model fibroblasts exhibit morphological features similar to those of cells *in vivo,* it is believed that the CM environment mimics the *in vivo* situation better than monolayer culture. Fibroblasts can contract the CM and this is considered to be a model for *in vivo* wound contraction (Finesmith et al., 1990; Germain et al., 1994). Three variations of this model are the anchored, floating and stress-relaxed matrices, which differ in physical properties. The morphology and proliferation of the embedded fibroblasts differ depending on whether the matrix is floating or anchored. In anchored matrix fibroblasts are under stress, exhibit polygonal to bipolar morphology and continue to grow, while in floating matrix fibroblasts are relaxed, exhibit spherical morphology and stop growing (Schor 1980; Harris et al., 1992; Nishiyama et al., 1993). Of particular interest in relation to aberrant wound healing are the alterations in fibroblast morphology, growth and survival and effects on TGF-ß and collagenase gene expression. I have already shown that re-organization of the cytoskeleton regulates expression of genes for TGF-ß1 and ECM proteins in dermal fibroblasts (Varedi et al., 1995; 1997; Chapters II and III). In previous work alterations in cell morphology were induced by trypsin, EGTA or cytochalasin C. Taking advantage of the fibroblast populated CM models in which re-organization of the cytoskeleton is influenced by mechanical forces rather than chemical agents which may have undesired side effects, I have investigated the expression of the mRNA for TGF-ß1, collagenase and type I collagen. I have also examined the effect of the mechanical stress-induced alterations in cell morphology on the production of TGF-ß1 protein, growth and survival of fibroblasts. These results show that the mechanical status of the CM has remarkable effects on morphology, expression of TGF-ß, collagenase, growth and survival of fibroblasts, factors which all play significant roles in the homeostasis of connective tissue.

MATERIAL AND METHODS

Materials

Type I collagen was prepared from fetal bovine skin and purified by repeated salt-precipitation according to Volpin and Veis (1971). Rabbit anti-TGF-ß1 antibody (100 µg/ml) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The second antibody, biotinylated goat anti rabbit IgG, and the Vectastain ABC Kit were purchased from Vector Laboratories Inc (Burlingame, CA). Proteinase K, dATP, dCTP, dGTP and terminal deoxynucleotide transferase (TdT) were purchased from GIBCO BRL (Grand Island, NY). Biotin-16-dUTP was purchased from Boehringer Mannheim (Germany). Bovine serum albumin (BSA), peroxidase-conjugated ExtrAvidin, normal non-immune goat and rabbit serum, bacterial collagenase type IV, pepstatin, phenylmethylsulfonyl fluoride (PMSF) and rhodamine-labeled phalloidin were purchased from Sigma Chemical Co. (St. Louis, MO). The complementary DNA (cDNA) probe for TGF-ß1 was characterized by Ardinger et al. (1988) and donated by Dr. G.I. Bell (Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, Illinois, USA). The type I procollagen ($\alpha1$) cDNA was characterized and provided by Drs. G. Tromp (1988) , H. Kuivaniemi (1988), and Ala-Kokko (1989, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA). The collagenase cDNA (Witham et al., 1986) and the mink-lung epithelial cells (Mv1Lu, CC1-64) were purchased from American Type Culture Collection (ATCC, 12301, Parklawn Drive, Rockville, MD, 20852, USA). The [α–^{32}P] dCTP was purchased from DuPont Inc. (Mississauga, Ontario). Nitrocellulose membrane (NitroPure) was purchased from MSI (Westboro, MA).

Cell Culture

Cultures of fibroblasts from dermis were established according to Nakano and Scott (1986) as described in Chapter II. The primary cultures were grown in Dulbecco's Modified Minimum Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and incubated at 37^{o} C in a humidified chamber under an atmosphere of 95% air and 5% CO_2. At confluence cells were harvested by a brief exposure to 0.1% (w/v) trypsin and 0.02% (w/v) EDTA and used for preparation of the fibroblast-populated collagen matrix (FPCM) and monolayer cultures on plastic. Cell cultures at passage 5 to 7 were used in this study.

Preparation of Fibroblast Populated Collagen Matrix (FPCM)

Fibroblasts were embedded within type I collagen according to a method modified from Bell et al. (1979). Briefly, fibroblasts were mixed with cold (12^{o}C) neutralized collagen in DMEM and 10% (v/v) FBS at a concentration of 1 - 2 x 10^{5}/ml. The collagen was originally dissolved in 0.01% acetic acid (3 mg/ml) and the final concentration in FPCM solution was 0.5 mg/ml. The suspension was mixed throughly and added to the culture plates. When total RNA was to be extracted, large-scale FPCMs were prepared by adding 35 - 40 ml of FPCM solution to 154 cm^{2} tissue culture plates. Smaller FPCMs, 2 ml/well in 12-well plates, were prepared for the TGF-ß extraction experiments. For staining with rhodamine-labeled phalloidin, immunohistochemistry or terminal deoxynucleotide transferase-mediated biotin-dUTP end labeling (TUNEL) assay, FPCM was prepared by adding 0.5 to 1 ml to each in 24-well plates. The FPCMs were incubated at 37° C in a 5% CO_2 and 95% air atmosphere. After polymerization, the FPCMs were either allowed to remain attached to the plate (ACM) or detached (FCM) with a sterile spatula. To make sure that the FCMs remained floating and the ACMs remained attached during the experiment, large FPCMs received 20 ml of fresh medium at this time. All FPCMs were incubated for 96 h. The stress-relaxed FPCM (St.rCM) were prepared by releasing the ACMs from the plate after 90 h. Monolayer cultures were prepared at the same time by plating an equivalent number of cells into culture flasks and grown under the same conditions.

Visualization of Cellular Actin Filaments

The organization of cytoskeleton actin filaments was visualized by staining with rhodamine-labeled phalloidin. Briefly, the FPCMs were washed in PBS and fixed in 4% (w/v) PBS-buffered paraformaldehyde for 3 h at room temperature or for 18 h at 4° C. The FPCMs were then washed in PBS, treated with 0.05% (v/v) Tween 20 in PBS, rewashed in PBS and incubated with a 1/200 dilution of stock solution of tetra-rhodamine isothiocyanide-labeled phalloidin (50 µg/ml 70% ethanol) for 15 min in the dark at room temperature. After washing in PBS, FPCMs were mounted on glass slides with 90% glycerol in PBS and examined under a fluorescence microscope.

RNA Extraction and Northern Analysis

The FPCM were transferred into a fresh plate, washed in PBS and digested by 5 ml of 4% (w/v) bacterial collagenase in DMEM for 15 min at 37° C with vigorous shaking to release the cells. The collagenase activity was stopped by adding 45 ml of ice-cold PBS. The cells were collected by centrifugation for 10 min at 4°C. The pellets were then lysed in 6 ml of 4 M guanidinium isothiocyanate (GITC) solution. The monolayer cultures were harvested by trypsinization, washed with ice-cold PBS and lysed in the same volume of GITC. Total RNA was then isolated by the GITC/CsCl procedure of Chirgwin et al. (1979) as described in Chapter II. The total RNA was then separated by electrophoresis (10 µg/lane) on a 1% (w/v) agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filters. To ensure that the gel lanes were loaded with similar amounts of total RNA, the quantities of 28S and 18S ribosomal RNA were compared visually by ethidium bromide fluorescence before and after blotting. The blots were baked for 2 h at 80^{o} C under a vacuum and then pre-hybridized for 4 h at 42^{o} C in a prehybridization solution. Hybridization was performed at 42^{o} C in the same solution for 16-20 h using either human TGF-ß1, type I procollagen (α1) or collagenase cDNA probes. The cDNA probes were labeled with ^{32}P-alpha-dCTP by nick-translation. The filters were initially washed at room temperature with 2 x SSC and 0.1% (w/v) SDS for 1 h and finally washed for 30 min at 65^{o} C in 0.2 x SSC and 0.1% (w/v) SDS. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at -70^{o} C in the presence of an intensifying screen. Autoradiograms and the corresponding ethidium bromide fluorescence photographs were scanned using a Color One Scanner (Macintosh, USA) and the quantitative analysis was accomplished using NIH Image software (Version 1.75). Loading of total RNA was also normalized using the fluorescence intensity of the ethidium bromide-stained 28 S band.

Acid-Ethanol Extraction of TGF-ß From FPCM

The TGF-ß was extracted from FPCM according to Roberts et al. (1980). Briefly, FPCM and the accumulated media were collected into a 15 ml tube containing 3 ml of a solution consisting of 37.5 ml of 95% ethanol, 0.75 ml of concentrated HCl and protease inhibitors (3.3 mg of PMSF and 0.19 mg of pepstatin). All steps were carried out at 4° C unless otherwise mentioned. The FPCM was immediately homogenized on ice for 2-3 min using a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y). After adding a further 2 ml of acid-ethanol solution, the mixture was mixed by vortex and extracted for 16 h. The samples were then centrifuged at 7,000 g for 30 min and the supernatant was collected into a fresh tube and stored at 4° C. The pellet was then re-suspended in 2 ml of acid-ethanol solution and re-extracted for 4 h. After centrifugation, the supernatants were combined and the pH was adjusted to 5.3 with 1 M ammonium hydroxide at room temperature. The undesired proteins were precipitated by adding 3 volume of 95% ethanol at -20° C for 48 h. The precipitate was then removed by centrifugation at 7,000 g for 30 min and discarded. The clear supernatant was extensively dialyzed against 0.1 M acetic acid using Spectrapor tubing (molecular weight cutoff 3500, Spectrum Medical Industries, Los Angeles, CA), stored at -80° C for few hours, briefly frozen in liquid nitrogen and lyophilized to dryness. The extracted TGF-ß was redissolved in 2 ml of DMEM, stored at -70° C and used in the mink lung epithelial cell growth inhibition assay.

Immunohistochemistry

A Vectastain ABC kit was used to localize and detect TGF-ß1 protein by immunohistochemistry in the FPCMs. The FPCMs were washed in PBS and fixed in 4% PBS-buffered paraformaldehyde for 18 h at 4° C. The FPCMs were then processed in an automatic processor and embedded in paraffin according to routine procedures. Sections of 4 µm thickness were mounted on Aptex-coated slides and maintained at room temperature. After deparaffinization and rehydration, sections were washed twice in PBS for 5 min each and endogenous peroxidase was blocked with 4% hydrogen peroxide (30%) in 50% (v/v) methanol solution. After permeabilization with 0.05% (v/v) Tween 20 in PBS, nonspecific binding was blocked with 10% (v/v) normal goat serum in PBS. The sections were then treated with the primary antibody, rabbit anti TGF-ß1 (1/100 dilution), for 24 h at 4° C in a humidified chamber. The sections were then washed in PBS and treated with the second antibody, biotinylated goat anti- rabbit IgG. After washing, the sections were treated with ABC reagent according to the manufacturer's instructions and stained with 3-3'diaminobenzidine solution and hydrogen peroxide and lightly counterstained with hematoxylin. In negative controls, primary antibody was replaced with non-immune normal rabbit IgG.

Mink Lung Epithelial Cell Growth Inhibition Assay

The assay was conducted according to a protocol modified from that originally described by Danielpour et al. (1989). Briefly, subconfluent cultures were trypsinized, washed in assay medium (DMEM, 0.2% v/v FBS, 10 mM HEPES and 1% v/v penicillin/ streptomycin antibiotic (GIBCO), seeded at 5 x 10^5/0.5 ml per well in 24 well plates and incubated for 6 h at 37° C in an atmosphere of 5% CO_2. At the end of this incubation period, the samples or standard amounts of TGF-ß1 were added, and 22 h later cells were labelled with 0.25 µCi of ^{3}H-labeled thymidine per well for 4 h at 37° C. Cells were then fixed and washed and the radioactivity was measured in the cell lysate collected from each well. All samples were assayed in triplicate. The standard curve was obtained with dilutions (80 - 0.5 pM) of the TGF-ß standard to convert the inhibition of DNA synthesis into concentration of TGF-ß. The levels of TGF-ß in conditioned media were calculated using the corresponding standard curve.

In Situ Detection of Apoptotic Cells by TdT-mediated biotin-16-dUTP Nick End Labeling (TUNEL)

The sections were prepared as described for immunohistochemistry. After a brief wash in ddH_2O, sections were pretreated with 2 x SSC for 20-30 min at 80^0 C. Slides were then washed in ddH_2O and the nuclei were stripped by incubation with proteinase K (20 µg/ml of 10 mM Tris, pH 8.00) at 37^o C. At the end of the incubation period, sections were washed in water and rinsed in reaction buffer (50 mM Tris-HCl, 5 mM MgCl, 10 mM ß-mercaptoethanol, and 0.005% BSA w/v, pH 7.5) . Sections were then dried and the transferase reaction was accomplished by incubation with TdT buffer (GIBCO- BRL) containing 0.01 mM dATP, dCTP, dGTP, 0.01 mM biotin-16-dUTP and 150 U/ml of terminal deoxynucleotidyl transferase (TdT) for 1.5 h at 15^o C and 0.5 h at 37^o C. The reaction was terminated by transferring the slides to 2x SSC. The endogenous peroxidase was destroyed by washing in PBS + 0.3% (w/v) H_2O_2 for 15 min at room temperature. Sections were then washed in PBS and nonspecific binding was blocked by 4% (w/v) BSA and 0.05% (v/v) Tween 20. After washing in PBS, sections were treated with peroxidase-conjugated ExtrAvidin for 30 min at room temperature. At the end of this incubation period, slides were washed with PBS and stained with 3-3' diaminobenzidine (DAB) solution (5% in 100 mM Tris buffer) and hydrogen peroxide (0.03%), and lightly counterstained with hematoxylin. Two or four sections from two different cell strains, were then dehydrated, mounted and the apoptotic cells were counted in 10 randomly selected fields (x 400 magnification) in a light microscope. In the negative control, TdT was replaced with ddH_2O and the positive control sections were treated with 200 µg/ml of DNase for 45 min at 37^{o} C before the transferase reaction.

Statistical Analysis

Wherever applicable, the data were analyzed by a paired two-tailed Student's t-test to determine if differences were significant ($p \leq 0.05$).

RESULTS

The Organization of Cytoskeleton Microfilaments is Influenced by The Nature and The Mechanical Status of The Matrix

Figure IV-1 shows the rhodamine-labeled phalloidin staining of filamentous actin in fibroblasts seeded on plastic or into different forms of CM. Fibroblasts were allowed to adhere and re-organize their cytoskeleton and remodel the CM for 96 h. Cells in ACM displayed a three-dimensional configuration ranging from bipolar to polygonal and exhibited well-defined filamentous actin organized into stress fibers (Figure IV-1a). In contrast the majority of cells in FCM displayed a more spherical configuration with long processes (filopodia) projecting in all directions. There was no sign of organized filamentous actin or stress fibers. Aggregates of actin were stained in a dot-like pattern along the filopodia and in the cell periphery and the cell body showed a diffuse pattern of staining (Figure IV-1b). The alterations in cell morphology were examined in St.rCM as soon as the FPCM was dislodged from the dish, since a marked reduction in the diameter of CM was observed immediately. Changes in cell morphology were observed within a few minutes. The cells started to retract their filopodia towards the cell bodies, the cytoskeleton microfilaments collapsed very rapidly and the stress fibers started to disintegrate. Four hours later, there was almost no sign of these fibers. The actin filaments were stained in these cells in a dot-like pattern (Figure IV-1c) similar to that of cytochalasin-treated cells. In the vicinity of the cells a number of small vesicular bodies were intensely stained. It is likely that these vesicles were budding off from the cells since some of them could be detected in association with the cell membrane at high magnification. After 24 h, cells displayed a similar shape and organization of microfilaments to that of cells in FCM (data not shown).

Cells grown on plastic for 24 h or longer exhibited well-developed stress fibers similar to those in cells grown in ACM (Figure IV-1d).

The Nature and Mechanical Status of Matrix Regulate Expression of TGF-ß1 As shown in Figure IV-2, the level of mRNA for TGF-ß1 is affected by the nature and re-organization of the substratum. Regardless of the model, cells grown in CM contained more mRNA for TGF-ß1 compared to cells grown in a rigid substratum (plastic). A significant increase was observed when I compared the level of TGF-ß1 mRNA in cells grown in either St.rCM (174 ± 30 vs 79 ± 30, p=.02) or FCM (284 ± 30 vs 79 ± 35, p=0.004) with that of cells grown on plastic. Although the level of TGF-ß1 mRNA in cells grown in ACM was also higher than that of cells grown on plastic, the difference was only marginally significant (125 ± 30 vs 79 ± 18, p=0.06). The mechanical status of the CM also influenced expression of mRNA for TGF-ß1. Cells grown in FCM expressed significantly higher levels of TGF-ß1 mRNA compared to cells grown in ACM (284 ± 35 vs 125 ± 18, p=0.009) or St.rCM (284 ± 35 vs 174 ± 12, p=0.05).

Levels of TGF-ß protein were assessed by immuno-histochemistry and Mv1Lu growth inhibition assay. As shown in Figure IV-3, cells grown in ACM (Figure IV-3a), FCM (Figure IV-3b) or St.rCM (Figure IV-3c) were immunoreactive for TGF-ß1. Although there was a considerable heterogeneity in the intensity of the staining, the majority of cells were positive. Consistent with the previous report, cells with different morphology showed a dramatic difference in the expression of immunoreactive TGF-ß1 and cells grown on plastic for 96 h expressed little or no immunoreactive TGF-ß1 (Figure IV-3d). Table IV- 1 shows the level of TGF-ß protein produced by cells grown in different models of CM assessed by the Mv1Lu growth inhibition assay. Cells grown in FCM produced 2 - 5 fold more TGF-ß than cells grown in ACM. Cells grown in St.rCM showed a 1.7 fold increase in TGF-ß production compared to cells grown in ACM even though these were allowed to relax for only a short time (6 h). Although there was variability between cell strains in TGF-ß production, the pattern of the response to stress-relaxation or the retraction of the CM was the same. Previously, using this assay no TGF-ß could be detected in conditioned medium from the cultures on plastic for 96 h (confluent culture).

The Nature and the Mechanical Status of Matrix Regulates Cell Growth and Survival Cells grown on plastic showed the highest rate of proliferation compared to other culture models tested. Cells grown on plastic apparently underwent about two doublings (400 ± 9% of 0 time) during 96 h. Although cell proliferation was repressed in ACM compared to that on plastic, cells continued to proliferate and apparently underwent about one doubling during the same period of time (183 ± 6% of 0 time). During 96 h, cells grown in FCM decreased in number (87 ± 5% of 0 time). Some of the fibroblasts grown in FCM or St.rCM exhibited morphological features of apoptotic cells such as dramatic shrinkage and condensation of the nuclei. Therefore, I proposed that fibroblasts may undergo programmed cell death, apoptosis, in this model which mimics *in vivo* wound contraction. As judged by the TUNEL assay, 5.7% (40/700 cells) of the cells grown in FCM (Figure IV-4b) were apoptotic while no apoptotic cells were observed in ACM (Figure IV-4a) or monolayer cultures (Figure IV- 4d). Two pre cent (8/398 cells) of the cells grown in St.rCM (Figure IV-4c) were also apoptotic. Apoptotic cells were round and appeared to be shrinking with no filopodia. Moreover, the nuclei in apoptotic cells were different in St.rCM and FCM. In FCM the nuclei of the apoptotic cells were small and fragmented with no defined morphology, while in St.rCM they were larger, unfragmented and round. It seemed that some cells in St.rCM were in the early stage of apoptosis and those in FCM were in the later stage.

Collagen Substratum Induces Expression of Collagenase but Not Procollagen I (α1) mRNAs As judged by Northern blot analysis of total RNA isolated from FPCMs, CM substratum induced a significant increase in the expression of mRNA for collagenase in cells grown in

ACM (196 ± 58 vs 74 ± 43, p=0.02), St.rCM (236 ± 53 vs 74 ± 43, p=0.005) and FCM (227 ± 34 vs 74 ± 43, p=0.003) as compared to plastic (Figure IV-5). The mechanical status of the CM seems not to have a significant effect on the expression of collagenase mRNA. As shown in Figure IV-6, the CM substratum did not have a significant influence on the level of mRNA for procollagen I (α1). Although the level of mRNA was moderately increased in FCM, the difference was not statistically significant.

DISCUSSION

I have previously reported that alterations in cell morphology and re-organization of cytoskeleton in human dermal fibroblasts regulate expression of genes for proteins involved in the structure, turnover and regulation of ECM (Varedi et al., 1995; 1997; Chapters II and III). However, in previous study monolayer cultures of fibroblasts on plastic were used and alterations in cell morphology were induced by cytoskeleton disrupting agents. In this study I took advantage of the *in vitro* remodeling of type I collagen matrix by fibroblasts, which is permissive for, or induces, changes in cell morphology. These results demonstrate that the remodeling of CM, one of the major events in many pathological and physiological processes including tissue repair, can modulate cell shape, expression of TGF-ß and collagenase, growth, and apoptosis in dermal fibroblasts.

The expression of TGF-ß1 was superinduced when fibroblasts were allowed to contract the CM. Since the organization of the cytoskeleton and cell morphology were profoundly affected by remodeling of CM, I believe that this induction is mediated through the mechano-transducers that regulate gene expression (Streuli et al., 1993; Roskelley et al., 1994). Consistent with the data presented in Chapter II, fibroblasts with different shapes showed differences in the expression of TGF-ß1 protein as judged by immunohistochemistry. Unfortunately, it is not possible to determine the actual cell shape or the organization of the cytoskeleton microfilaments in paraffin sections. Therefore, I could not study the correlation between the level of expression of TGF-ß1 and specific morphology or pattern of the cytoskeleton microfilaments. Surprisingly, the elevated levels of TGF-ß were associated with the presence of apoptotic cells when fibroblasts were stress-relaxed and allowed to contract the matrix. Shortly after relaxation, fibroblasts exhibited a morphology similar to that of cytochalasin-treated cells (Chapter II, Figure II-2). It has been reported that cytochalasin induces apoptosis in several cell types (Scott et al., 1997; Peralta Soler et al., 1996; Sauman and Berry, 1993) and increases the expression of TGF-ß mRNA in dermal fibroblasts (Varedi et al., 1995; 1997; Chapters II and III), suggesting that intact cytoskeleton microfilaments are involved in transduction of survival signals and that TGF-ß may be involved in the apoptotic response. Although the growth inhibitory and apoptotic effects of TGF-ß on epithelial and immune cells are well documented (Nass et al., 1996;), its effects on the growth and survival of fibroblasts are still controversial (Lyons and Moses, 1990). The stimulatory effect of TGF-ß on the growth of mesenchymal cells is believed to be indirect (Lyons and Moses, 1990). However, the involvement of TGF-ß in fibroplasia is generally accepted (Border and Ruoslahti, 1992).

Several studies, including this work, have shown that fibroblasts change in morphology and become growth arrested when allowed to contract their matrix (Schor, 1980; Harris et al., 1992; Nishiyama et al., 1993). However, an effect on the survival of fibroblasts has not been previously reported. A reduction in cell number in contracted CM suggests that remodeling can control survival of fibroblastic cells. Programmed cell death or "apoptosis" is an essential process during embryogenesis and an important mechanism for the homeostasis of every higher organism (Ucker, 1992). The maintenance of cell number in the body is controlled by balancing cell proliferation with cell death. It has been suggested that proliferation of tumor cells is associated with blocking the apoptosis pathway (Reed, 1994). The role of tumor repressor genes in apoptosis is well documented and a number of cytokines have been shown to be involved in the modulation of the expression of these genes (Nass et al., 1996). Although most investigators have used epithelial, endothelial or immune cells to investigate the role of TGF-ß in apoptosis, Bauer and colleagues showed that the addition of exogenous TGF-ß to a co-culture of normal and transformed fibroblasts leads to a dramatic reduction of colony-forming transformed fibroblasts (Hofler et al., 1993). The elimination of transformed fibroblasts by their normal counterparts is mediated by induction of apoptosis (Jurgensmeier et al., 1994). Perhaps, one of the best *in vivo* examples of apoptosis in fibroblast is the elimination of myofibroblasts in granulation tissue during cutaneous wound healing (Desmouliere et al. 1995). Granulation tissue is known as a contractile structure that undergoes a massive reduction in cellularity during contraction/remodeling. It has been suggested that the inhibition of the elimination of fibroblasts in granulation tissue is responsible for the development of hypertrophic scars and keloid (Desmouliere et al., 1995), both characterized by hypercellularity and imbalanced deposition of ECM in injured dermis. Although a low level of collagenase activity may account in part for the excessive deposition of collagen in fibrotic lesions, a significant expression of collagenase has been reported in areas of scars which were undergoing active remodeling (Hembry and Ehrlich, 1986). Furthermore, it has been shown that the resolution of fibrosis is associated with a reduction in cellularity and collagen deposition, most likely by apoptosis and restoration of collagenase activity. Desmouliere et al. (1995) have reported a dramatic increase in the proportion of apoptotic fibroblasts and vascular cells during wound closure. However, the stimulus leading to apoptosis and restoration of collagenase expression has not been previously addressed.

The apparent failure of TGF-ß to affect expression of collagen and collagenase in fibroblasts grown in CMs, despite the high level could be due to the repression in the response to growth factors (Nishiyama et al., 1991) or the bioactivity of the TGF-ß. Moreover, the presence of certain species of proteoglycans (for example decorin) which are believed to neutralize TGF-ß activity (Yamaguchi et al., 1990; Hausser et al., 1994) may also affects the bioavailability of the TGF-ß in CMs. Quantitative evaluation of decorin expression in these models would be of value. The relationship between apoptosis and the high level of expression of TGF-ß in stress-relaxed fibroblasts (St.rCM and FCM) remains to be investigated.

CONCLUSION

The data presented in this chapter show that stress-relaxation/re-organization of matrix can trigger apoptosis and that the flexibility of matrix modulates expression of TGF-ß and collagenase. Stress could be developed when fibroblasts are attached to a rigid matrix. This may be the case in fibrotic lesions such as HSc. Recently, Scott et al. (1996) have quantified and characterized the proteoglycans in human post-burn hypertrophic and mature scars. They reported that the small dermatan sulphate proteoglycan, decorin, was lower and the large chondroitin sulphate proteoglycan, versican was much higher in HSc than in normal dermis and mature scar tissue. This group suggested that the abnormal architecture of the ECM in HSc is a consequence of the reduction in decorin which has profound effects on the organization of fibrillar collagen (Scott et al., 1993; 1988) and the over-hydrated matrix in HSc is a consequence of the high expression of versican that plays a significant role in tissue resilience (Zimmerman et al., 1994). These characteristics could account at least in part for the rigid and inelastic quality of HSc tissue which could place the fibroblasts under considerable mechanical stress. Maturation of HSc is associated with a return of the proteoglycan composition to that characteristic of normal dermis (Scott et al., 1995). This could relieve the stress on fibroblasts leading to apoptosis and a reduction in cellularity. At the same time restoration of flexibility of the matrix could induce collagenase expression, resulting in resolution of HSc. It is intriguing that animals in which wounds close and heal mainly by contraction, i.e. those with mobile skin, do not develop hypertrophic scar and that the wounds which have been open (failed to contract) for a long time are prone to the development of HSc. Based on these results, I suggest that physicochemical properties of the ECM and stress-relaxation play determinant roles in the outcome of wound healing process and are important in resolution of fibrotic lesions.

Figure IV-1:

The nature and re-organization of ECM induce alterations in cell shape and organization of filamentous actin in dermal fibroblasts. Micrographs showing shape and the organization of actin filaments in whole mounted FPCM and monolayer cultures visualized by rhodamine-labeled phalloidin. Cells grown in ACM (a) or on plastic (d) show polygonal or bipolar morphology and stress-fibers. Cells in FCM(b) exhibit a round body, long filopodia and an undefined organization of actin filaments. Cells in St.rCM (c) present a collapsed cytoskeleton 4 h after dislodgment.

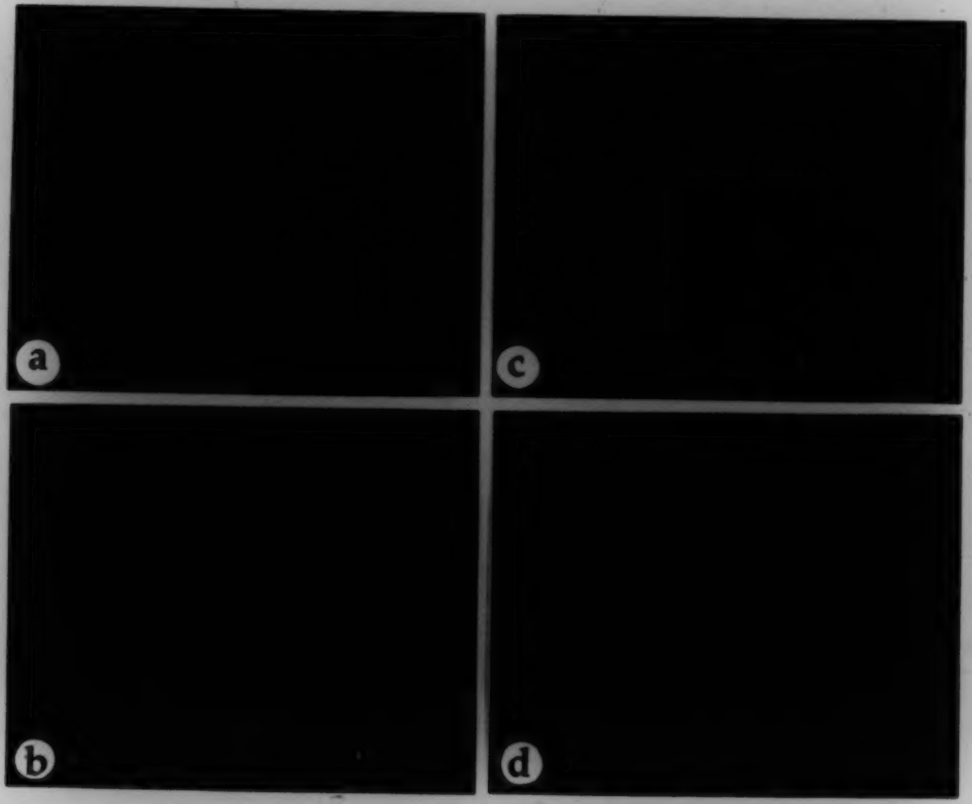


Figure IV-2:

The nature and re-organization of ECM regulate expression of TGF-ß1 mRNA. A) Representative Northern blot analysis of TGF-ß1 mRNA (2.5 Kb) from dermal fibroblasts grown on plastic (P) or in ACM, St.rCM and FCM. B) Fluorescence photograph of the ethidium bromide staining pattern of the original gel. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 individually tested cell strains. * Statistically significant (p≤0.05).

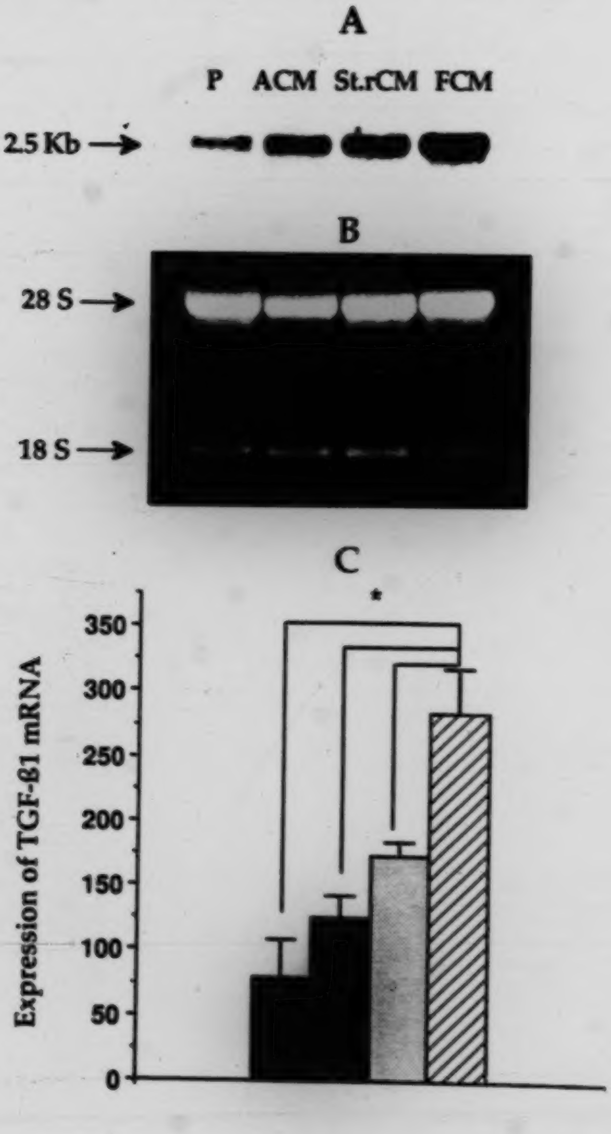


Figure IV-3:

The nature and re-organization of ECM modulate expression of immunoreactive TGF-ß1. Immunohistochemical staining of 4 μm sections of FPCMs or the whole monolayer cultures. Cells were grown in ACM (a), FCM (b), St.rCM (c), or on plastic (d), fixed and processed as described in materials and methods. Cells grown in CM (a, b, c) exhibit a considerable heterogeneity in cell morphology and the levels of immunoreactive TGF-ß1 while most are positive. Cells in monolayer culture (d) show little or no immunoreactive TGF-ß1.

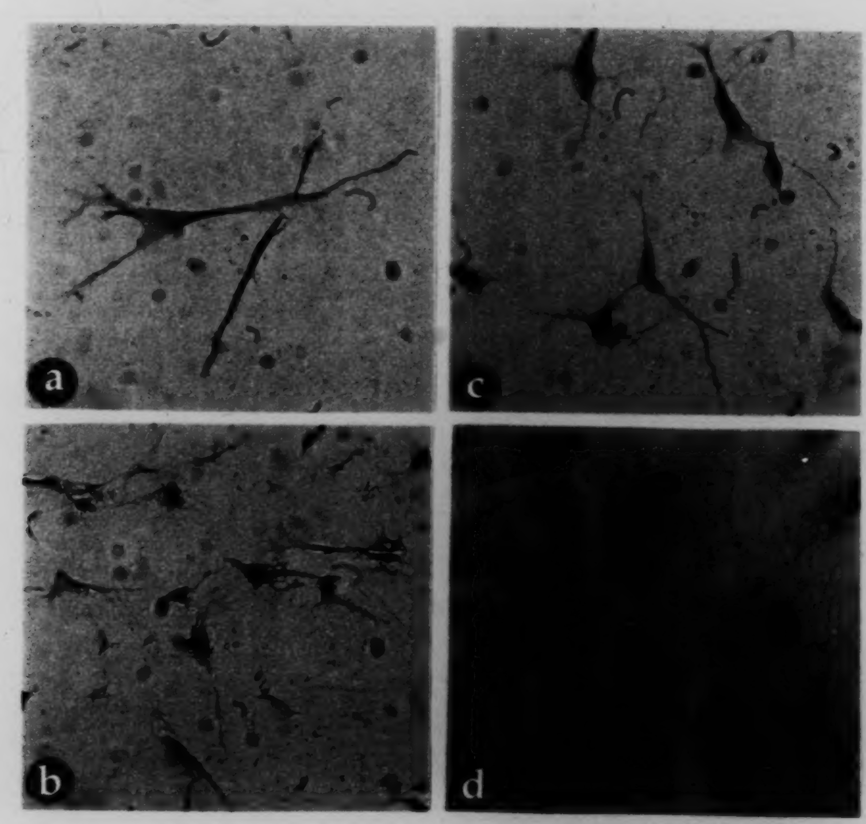


Figure IV- 4:

Stress-relaxation triggers apoptosis in dermal fibroblasts. TUNEL of sections (4 μm) of FPCMs and the whole monolayer fibroblast culture. a) ACM, b) St.rCM, c) FCM, and d) monolayer culture. The arrows indicate the apoptotic cells. No apoptotic cells were seen in ACM or monolayer cultures.

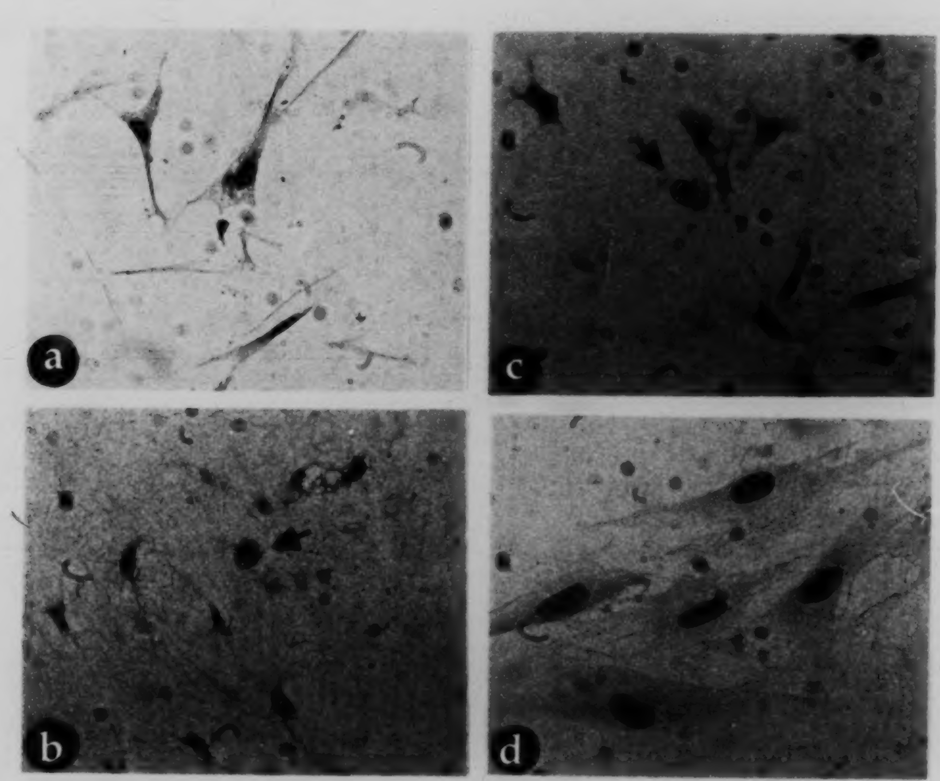
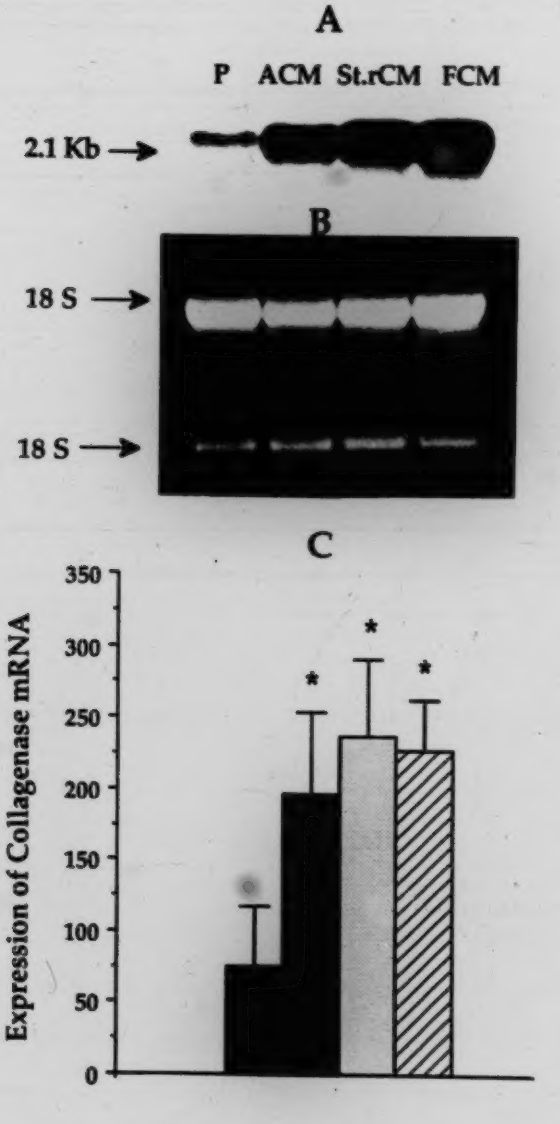


Figure IV- 5:

The nature of ECM regulates the expression of collagenase mRNA. A) Representative Northern blot analysis of collagenase mRNA (2.1 Kb) from dermal fibroblasts grown on plastic (P) or in ACM, St.rCM and FCM. B) Fluorescence photograph of the ethidium bromide staining pattern of the original gel. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 individually tested cell strains. * Statistically significant (p≤0.05).



IV- 6:

The expression of procollagen I (α1) mRNAs is not affected by the nature or re-organization of CM. A) Representative Northern blot analysis of procollagen I (α1) mRNAs (5.8 Kb and 4.8 Kb) from dermal fibroblasts grown into ACM, St.rCM, FCM or on plastic (P). B) Fluorescence photograph of the ethidium bromide staining pattern of the original gel. C) Quantitative analysis of the autoradiograms. Each bar shows the mean ± SEM of 4 individually tested cell strains.

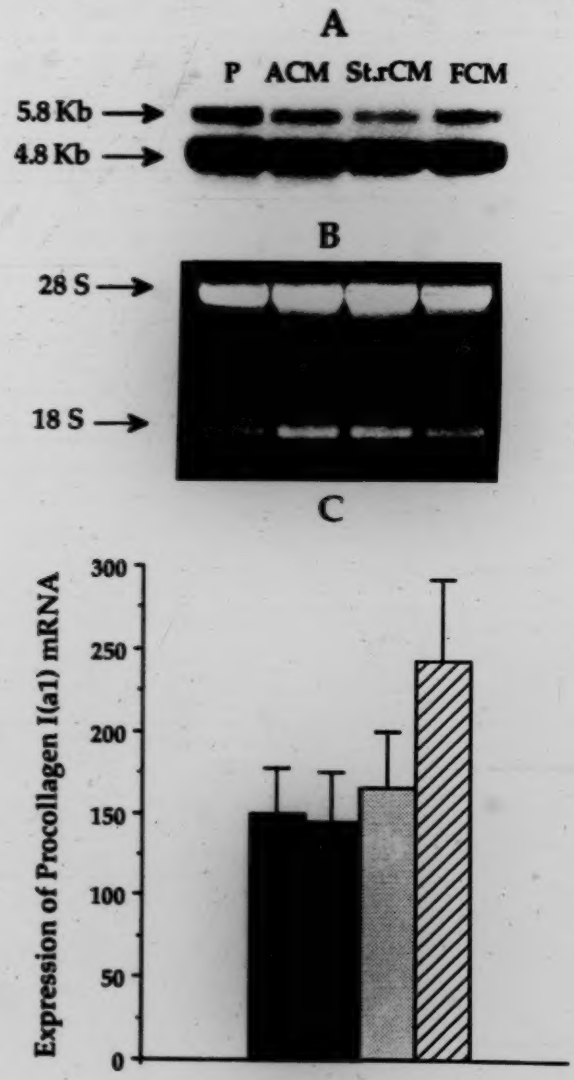


Table IV-1:

Re-organization of CM induces production of TGF-ß in dermal fibroblasts. Cells were grown in ACM, St.rCM and FCM for 96 h. TGF-ß were extracted as described in materials and methods and the levels were evaluated by Mv1Lu growth inhibition assay. Each value is the mean of 2 experiments ± range. The levels of TGF-ß in each extract has been measured in triplicate. The levels of TGF-ß are calculated as described in materials and methods. Three different cell strains (C1-C3) have been tested.

TGF-ß Protein (ng/10^6cells)

Cell Strain	ACM	St.rCM	FCM
C1	13 ± 3	30 ±10	45 ± 22
C2	166 ± 18	208 ±17	455 ± 58
C3	82 ± 14	143 ± 25	446 ± 49

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V

GENERAL DISCUSSION AND CONCLUSIONS

DISCUSSION

During many physiological and pathological processes including cutaneous wound healing and hypertrophic scarring (HSc), the extracellular matrix (ECM) undergoes a series of changes in the physicochemical properties that subsequently affect the morphology of the resident cells, particularly fibroblasts. Fibroblasts are the major cell population in the dermis and are responsible for the deposition, maintenance and turnover of the ECM components. These cells migrate to the wound site soon after tissue injury and begin to proliferate, assist other phagocytes in scavenging the debris, and repair the damaged tissue. Therefore, they play a central role in tissue repair. The aberrant activity of fibroblasts can impair healing and/or lead to development of abnormal scars. Hypertrophic scarring is known as a fibroproliferative disorder that frequently develops as a result of deep dermal injuries and usually undergoes spontaneous resolution (Ross, 1987). However, the scars are usually associated with tissue deformation and contractures which often impairs organ function. Although age, race, prolongation of the inflammatory phase of wound healing and the site of injury are known as risk factors, the etiology of these fibrotic lesions is not yet known. Evaluation of the available data on the pathogenesis of fibrotic disorders suggests a failure in the ECM metabolism. Hypertrophic scar tissue is characterized by abnormal composition and organization of the ECM and the presence of activated fibroblasts, i.e. those with aberrant morphology and activity (Kischer et al., 1982; 1990). It was the aim of this thesis to examine the effects of alterations in cell morphology and re-organization of the matrix on fibroblast activity. Of particular interest was the correlation between alterations in cell morphology and gene expression for the proteins involved in the structure (type I collagen and fibronectin), turnover (collagenase and TIMP-I) and regulation (TGF-ß1) of ECM, and growth and survival of dermal fibroblasts.

Alterations in morphology of the cells grown in monolayer culture on plastic were induced by disruption of the cytoskeleton using three different agents: trypsin, EGTA or cytochalasin C (Chs), which alter cell morphology through different mechanisms (Rees et al., 1977; Yahara et al., 1982). Visualization of the cytoskeleton microfilaments by rhodamine-labeled phalloidin (Cooper, 1987) confirmed that the morphology and organization of the cytoskeleton of the cells treated with cytochalasin were different from those of trypsin- or EGTA-treated cells. Fibroblasts harvested by trypsin or EGTA and replated for a short time may resemble migratory, dividing or differentiating cells, reorganizing the cytoskeleton after disruption by loss of cell-cell and/or cell-matrix interactions (Rees et al., 1977). Alterations in cell morphology induced by Chs may simulate *in vivo* situations in which the intracellular activity of capping proteins is changed and the cytoskeleton microfilaments are transiently disassembled (Ben-Ze'ev, 1991), but the cells are still in touch with the matrix (Yahara et al., 1982). The results from Northern analysis and nuclear run-on assays from chapters II and III indicated that re-organization/disorganization of the cytoskeleton was associated with increases in the rate of gene transcription and the levels of mRNA for TGF-ß1 and collagenase, regardless of the type of stimulus, while the gene transcription and the levels of mRNA for type I collagen, fibronectin and TIMP-I were selectively modulated. A significant increase in the level of mRNA but not the rate of transcription of the gene for TIMP-I, and a marked increase in the transcription rate but not the levels of mRNA for type I collagen and fibronectin in Chs-treated cells, indicated that the status of the cytoskeleton may also affect the expression of the proteins involved in ECM metabolism by controlling the half-life of the mRNA. Taken together, the data suggest that expression of the genes for TGF-ß and the proteins involved in ECM metabolism are selectively regulated by the status of the cytoskeleton which undergoes alterations during cell division, migration and differentiation, events which are fundamental to wound healing and tissue remodeling.

The purpose of the research described in chapter IV was to examine the effects of alterations in cell morphology on fibroblast activity in an environment which mimics the *in vivo* situation better than monolayer culture on plastic. An *in vitro* model of fibroblast culture in a biologic matrix, type I collagen (CM), was utilized (Bell et al., 1979). Another important feature of collagen matrix is that it can be physically re-organized by the cultured fibroblasts (Frey et al., 1995). Therefore, the effects of the remodeling of matrix (contraction), a critical event in wound healing process, which could not be addressed in the studies using monolayer cultures on plastic, were also investigated. The levels of expression of the genes of interest as well as the growth and apoptosis of fibroblasts were evaluated in three variations of this model; namely anchored (ACM), stress-relaxed (St.rCM) and floating (FCM) matrices. The mechanical status of these matrices differs depending on whether they are floating or anchored. Taking advantage of this characteristic, alterations in cell morphology were induced by allowing fibroblasts to re-organize (contract) the matrix soon after seeding or by stress-relaxation of the fibroblasts initially seeded in an anchored matrix for about 4 days and then dislodged for a few hours. Visualization of the cytoskeleton microfilaments confirmed that both re-organization (contraction) of the matrix and stress-relaxation of fibroblasts have profound effects on the morphology of cultured fibroblasts. Consistent with the experimental data from Chapters II and

III, alterations in cell morphology following re-organization of the matrix or stress-relaxation also selectively modulated the expression of TGF-ß and the proteins involved in ECM metabolism. Although the CM stimulated expression of TGF-ß mRNA and protein, the effects of the remodeling were more pronounced, indicating that TGF-ß may play a significant role in tissue remodeling. A higher level of immunoreactive TGF-ß in mature scar tissue compared to HSc tissue reported by Scott et al. (1995) supports this idea. TGF-ß is believed to be a potent fibrogenic factor. The stimulatory effects of TGF-ß on the expression of ECM proteins and its inhibitory effects on matrix metalloproteinases have been reported (Ignotz et al., 1987; Edwards et al., 1987). The apparent failure of TGF-ß to affect expression of collagen and collagenase in CM cultures, despite the high level could possibly be due to the presence of ECM proteins, which are capable of binding and modulating many growth factors (Ruoslahti and Yamaguchi, 1991), or repression in the response to growth factors and/or bioactivity of the TGF-ß. Proteoglycans have been found to be one of the best binders and modulators of growth factors. Qualitative evaluation of the small dermatan sulfate proteoglycan, decorin, which binds and neutralizes TGF-ß (Yamaguchi et al., 1990) would be valuable. Whether the higher levels of immunoreactive TGF-ß in CM cultures compared to monolayer culture is due to the retention of TGF-ß by decorin and /or other matrix components remains to be investigated.

Evaluation of cell numbers of fibroblasts grown on plastic and different types of CM revealed that the nature and organization of the matrix control the growth activity and survival of fibroblasts. The experimental data presented in Chapter IV showed that fibroblasts grown on plastic (rigid matrix) have the highest rate of proliferation compared to the other tested culture systems. Fibroblasts in FCM culture not only stop growing but also undergo apoptotic cell death. Detection of apoptotic fibroblasts in St.rCM but not ACM suggests that stress-relaxation could trigger apoptosis. Stress could be developed when fibroblasts are attached to a rigid matrix, a characteristic of the ECM in HSc which may place the resident fibroblasts under mechanical stress. Inappropriate expression of proteoglycans in HSc is believed to be responsible for the inelastic quality of the ECM (Zimmerman et al., 1994, Scott et al., 1996). Moreover, inhibition of the elimination of fibroblasts by apoptosis during evolution of granulation tissue is believed to be responsible for the hypercellularity and imbalanced deposition of ECM and development of HSc (Desmouliere et al., 1995). It has been suggested that the resolution of HSc is dependent on a return of the level of the expression of proteoglycans to those characteristic of normal dermis (Scott et al., 1996) and the restoration of tissue elasticity.

A dramatic increase in the expression of collagenase mRNA in CM cultures indicated that elasticity of the matrix has a profound effect on the expression of this matrix metalloproteinase. Thus, restoration of the elasticity of the matrix could also induce the expression of collagenase, facilitating the resolution of HSc. It is intriguing that animals in which wounds close and heal mainly by contraction, i.e. those with mobile skin, do not develop HSc and that wounds which have been open (failed to contract) for a long time are prone to develop HSc. The growth inhibitory and apoptotic effects of TGF-ß in epithelial, endothelial and immune cells are well documented (Nass et al., 1996; Tsukada et al., 1995). However, its effects on the growth and survival of fibroblastic cells are still controversial (Lyons and Moses, 1990). As discussed in Chapter IV, the elevated levels of TGF-ß in CM cultures were associated with the presence of apoptotic cells when fibroblasts were allowed to contract the matrix. Whether TGF-ß plays a role in apoptosis of fibroblasts during wound contraction remains to be investigated.

CONCLUSIONS

The data presented in this thesis have revealed new insights into the activity of fibroblasts at the molecular level during wound healing and tissue remodeling. This work has also significantly advanced our understanding of the pathophysiology of wound healing and resolution of hypertrophic scars. Significant findings are:

I. upon activation (alterations in cell morphology) dermal fibroblasts can be a rich source of TGF-ß,

II. fibroblasts derived from normal dermis and hypertrophic scar tissue show similar responses to alterations in morphology,

III. the status of the cytoskeleton differentially modulates expression of the genes for TGF-ß1 and the proteins involved in ECM metabolism,

IV. disassembly of the cytoskeleton microfilaments modulates the stability of the mRNA for TGF-ß1, and proteins involved in ECM metabolism,

V. mechanical stress and re-organization of collagen matrix have profound effects on the morphology of fibroblasts,

VI. mechanical stress and re-organization of collagen matrix differentially modulate expression of the mRNA for TGF-ß1 and collagenase,

VII. the nature and/or elasticity of the matrix has significant effects on the proliferation of fibroblasts,

VIII. stress-relaxation and re-organization of collagen matrix modulates survival (apoptosis) in dermal fibroblasts.

LIMITATIONS AND NEW VISTAS

While *in vitro* investigations will most likely continue to provide a treasure of information, the road between the *in vivo* and *in vitro* activity of cells must be marked with a clear warning sign. Perhaps the warning sign for dermal fibroblasts should read: CAUTION: Isolated dermal fibroblasts in culture only tell us what they can do, not what they actually do when in the company of other cells in their own organ environment: skin. However, investigations into the role of fibroblasts in the pathogenesis of hypertrophic scarring are limited to *in vitro* cell culture systems. This is due to the fact that laboratory animals do not develop HSc.

Since 1979 when Bell et al. introduced fibroblast culture in type I collagen matrix, several models of fibroblasts culture in matrix and dermal equivalents have been developed for studying fibroblast activity in environments which are believed to mimic the *in vivo* situation better than monolayer culture on plastic. Despite the ability of the cultures to induce the *in vivo* morphological and growth characteristics of fibroblasts; they are certainly not optimal for many other activities, since they lack the variety of intricate cell-cell, cell-molecular and molecular-molecular interactions of the *in vivo* system. Addition of other cellular and molecular elements of dermis such as immune cells, endothelial cells, type III collagen, elastin, and different species of proteoglycans and a co-culture of different phenotypes of fibroblasts and epithelial cells may take the *in vitro* culture systems a few steps closer to the *in vivo* environment.

It is believed that a failure in the resolution of hypercellularity in the granulation tissue (inhibition of apoptosis) leads to development of HSc. Thus, it is important to investigate factors which are involved in regulation of the growth and apoptosis in fibroblasts during maturation of the granulation tissue. I have shown that the rigidity and mechanical stress-relaxation modulate growth/survival in dermal fibroblasts. It is important to investigate the phenomenon in an *in vivo* situation, perhaps in an animal model of wound contraction. Evaluation of the rate of growth/apoptosis of fibroblasts derived from normal dermis and HSc in response to mechanical stress-relaxation would be also valuable. The high level expression of TGF-ß and apoptosis after stress-relaxation is remarkable and the correlation can be investigated by blocking the bioactivity of TGF-ß or by adding exogenous TGF-ß to a culture system in which fibroblasts do not normally undergo apoptosis. Whether or not TGF-ßs play a central role in wound healing and hypertrophic scarring, I believe that the study of the effects of TGF-ßs/cytokines on fibroblast behavior will lead to a better understanding of the factors which control ECM homeostasis and to more successful treatments of chronic wounds and HSc.

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